Institutional Repository - Research Portal
Dépôt Institutionnel - Portail de la Recherche
researchportal.unamur.be
University of Namur

THESIS / THÈSE

DOCTOR OF SCIENCES

Understanding the link between osmoregulation and immunity in striped catfish Pangasianodon hypophthalmus (Sauvage)
Molecular and environmental perspectives

Schmitz, Mélodie

Award date:
2017

Awarding institution:
University of Namur

Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 05. May. 2023

FACULTY OF SCIENCES

DEPARTMENT OF BIOLOGY

RESEARCH UNIT IN ENVIRONMENTAL AND EVOLUTIONAL BIOLOGY (URBE)

Understanding the link between osmoregulation and immunity in striped catfish *Pangasianodon hypophthalmus* (Sauvage).

Molecular and environmental perspectives.

A dissertation submitted by

Mélodie SCHMITZ

in fulfillment of the requirements

for the degree of PhD in Biological Sciences.

January 2017

Members of the Examination Committee:

Patrick Kestemont (Supervicor, UNamur)

Jessica Douxfils (Co-supervisor, UNamur)

Eric Depiereux (President of the jury, UNamur)

Peter Bossier (UGent)

Eric Muraille (ULB)

Lluis Tort (UAB Barcelona)

Comprendre le lien entre l'osmorégulation et l'immunité chez le poisson-chat asiatique *Pangasianodon hypophthalmus* (Sauvage)

Approches moléculaire et environnementale

Par Mélodie SCHMITZ

RESUME

En 2014, la production de poisson-chat vietnamien atteignait 1.2 million de tonnes, pour une valeur nette d'exportation estimée à 1.77 milliard US$, représentant 80% de la production mondiale de cette espèce. Le Delta du Mekong, qui produit 77% des exportations aquacoles du pays, est actuellement menacé par l'avancée d'un front d'eau salée provenant de la Mer de Chine et du Golfe de Thailande et induit par l'augmentation du niveau de la mer et la construction de barrages en amont. Dans les fermes aquacoles de poisson-chat, des salinités dépassant les 10 ppt (un tiers de l'eau de mer) ont été mesurées en saison sèche et ont été corrélées à une diminution de la croissance et une augmentation de la mortalité dans les étangs. Des actions à court terme, telles que la construction de digues ou l'augmentation de l'apport d'eau du fleuve, ont été entreprises mais ne permettront pas d'assurer la protection à long terme des fermes aquacoles face aux changements de salinité. Ainsi, cette thèse a pour but premier d'étudier les conséquences d'un stress hyperosmotique chronique sur la physiologie du poisson-chat vietnamien, particulièrement sur le système immunitaire et la résistance aux maladies, dans le but d'évaluer les effets à long terme d'un stress osmotique sur la pérénité de la production aquacole de poisson-chat dans cette région. Au Vietnam, deux campagnes d'échantillonnage ont été réalisées en saison sèche 2013 (mars-avril) et humide 2014 (juillet-août) dans 12 fermes de poisson-chat localisées dans le Bas (Province de Tra Vinh) et le Haut (Province de Can Tho) du Delta du Mekong. Cette étude a permis de dresser un premier bilan relatif aux paramètres osmorégulateurs (osmolalité du plasma, activité NaK ATPase) et immunitaires (abondance et activité des cellules immunitaires, activité du complement et du lysozyme) du poisson-chat vietnamien en aquaculture en fonction de la localisation (proximité de l'embouchure) et des saisons (débit du fleuve).

Les effets d'un stress hyperosmotique sur le système immunitaire des poissons ont été relativement peu étudiés, les études passées se concentrant principalement sur le système osmorégulateur et la croissance. Chez le poisson, de nombreux auteurs ont pourtant démontré qu'un stress salin chronique pouvait induire la stimulation de paramètres de l'immunité innée tels que l'abondance et l'activité des cellules immunitaires, et interférer avec la résistance à certains pathogènes bactériens ou viraux. Chez les mammifères, les fluides hyperosmotiques (fluides de réanimation) sont largement utilisés afin de moduler le système immunitaire chez les patients après un trauma ou un choc hémorrhagique. Ainsi, le deuxième objectif de cette thèse visait à découvrir de nouveaux mécanismes impliqués dans la réponse osmotique, particulièrement ceux ayant un lien avec la réponse immunitaire. Dans ce but, des poissons-chats vietnamiens ont été soumis à trois profils différents de salinité (eau douce, 10 ppt, 20 ppt) durant 20 jours, ensuite contaminés à l'aide d'une souche bactérienne virulente (*Edwardsiella ictaluri*) couramment rencontrée en aquaculture et responsable d'une septicémie hémorrhagique chez les siluriformes. Divers facteurs osmorégulateurs (osmolalité et activité NaK ATPase) et immunitaires (abondance et activité des cellules immunitaires, activité du complément et du lysozyme, production de chaperones moléculaires, expression des Toll-Like Receptors) ont été étudiés. De plus, une étude protéomique sans gel a été effectuée dans le rein afin de mettre en évidence de nouvelles voies immunitaires et métaboliques affectées par un stress hyperosmotique.

Les poissons-chats vietnamiens exposés à un stress hyperosmotique voient leur osmolalité plasmatique augmenter tandis que l'activité des pompes NaK ATPase reste inchangée jusque 10 ppt. Chez des poissons non infectés ou infectés par la bactérie, la salinité a un effet stimulateur sur les divers paramètres de l'immunité innée testés. Cependant, les poissons soumis à un stress osmotique montrent en général une susceptibilité accrue face à la bactérie *E. ictaluri*. Dans le rein, la salinité et l'infection exercent une activité synergique sur l'abondance du protéome. L'activité métabolique (métabolisme lipidique, protéique, énergétique) ainsi que plusieurs mécanismes de détoxification sont régulés à la hausse chez les poissons exposés à la salinité. De nombreuses protéines structurales telles que l'actine, le collagène et la spectrine sont différentiellement exprimées en fonction de la pression osmotique. En eau saumâtre, l'abondance de plusieurs protéines impliquées dans la voie de signalisation MAPKp38, la maturation phagosomale et la régulation des lymphocytes T est stimulée. En eau salée, diverses protéines impliquées dans la réponse inflammatoire et la réponse au stress augmentent également en abondance. Cependant, l'abondance des transcrits relatifs à l'expression des Toll-like receptors (TLRs), à l'exception du TLR5, diminue chez les poissons stressés, particulièrement durant l'exposition à la bactérie.

En conclusion, un stress salin occasionne chez le poisson-chat vietnamien une augmentation de la pression osmotique interne, associée à une réponse inflammatoire, dont l'intensité varie en fonction de l'intensité du stresseur. Cette réponse inflammatoire interfère avec la réponse immunitaire lorsque l'animal est exposé à un pathogène (*Edwardsiella ictaluri* dans notre cas) et peut modifier l'interaction hôte-pathogène, au bénéfice de la bactérie lorsque la salinité est supérieure à 10 ppt. Cependant, dans quelle mesure le poisson contrôle-t-il la réponse inflammatoire induite par un stimulus osmotique et dans quelle mesure l'inflammation interagit-elle avec la réponse spécifique au pathogène sont autant de questions qui restent à investiguer.

Understanding the link between osmoregulation and immunity in striped catfish *Pangasianodon hypophthalmus* (Sauvage).

Molecular and environmental perspectives.

By Mélodie Schmitz

ABSTRACT

In Vietnam, striped catfish production reached 1.2 millions of tons in 2014 with an estimated value of 1.77 billion US$ in the international food market, representing almost 80 % of the total world production of this species. The Mekong Delta, which actually includes 77 % of fisheries Vietnamese production, is currently threatened by saltwater intrusion from the China Sea and the Gulf of Thailand induced by the sea-level rise and aggravated by dams construction upstream. In striped catfish ponds, salinity may increase up to more than 10 ppt (nearly one third of seawater) during the dry season and is correlated with decreasing growth rate and higher mortality rate. Short-term actions have been undertook by local authorities such as construction of dykes and increase in water exchange but will not resolve the problem of salinity in the long-term. This thesis deeps the consequences of saline stressors in striped catfish health, particularly the immune system and susceptibility to disease, in order to investigate the long-term possible effects of salinity on striped catfish' farms perenity. In Vietnam, a survey was carried out during the dry season 2013 (March-April) and rainy season 2014 (July-August) in 12 fish farms of the lower (Tra Vinh Province) and higher (Can Tho Province) Mekong River Delta. This survey allowed drawing up current key osmoregulatory (plasma osmolality and NaK ATPase activity) and innate immune factors (immune cells abundance and activity, lysozyme and complement activity) of striped catfish in aquaculture depending on the geographical location, particularly the proximity to the River Mekong Estuary and the seasonal surface runoff.

In fish, hyperosmolarity has often been investigated from osmoregulation perspectives while the effects of such stress on the immune capacity remain largely unexplored. However, in fish, several authors pointed out that chronic saline stressor may stimulate several innate immune factors such as immune cells proliferation and activity (e.g. lysozyme activity, respiratory burst, phagocytosis) and interfere with susceptibility of viral and bacterial diseases. In mammals, infusions of "resuscitation fluids" (hyperosmotic fluids) have found a lot of clinical applications in order to modulate the immune system in patients after hemorrhagic shock or trauma. Therefore, the second objective of this thesis was to point out new immune mechanisms modified by chronic hyperosmolarity and study the link between osmoregulation and immunity. Hence, striped catfish were submitted to three salinity profiles (freshwater, 10 ppt, 20 ppt) during 20 days in husbandry conditions, followed by infection with a virulent bacteria, *Edwardsiella ictaluri*, responsible for the enteric septicemia of catfish. Osmoregulatory factors (plasma osmolality, gill NaK ATPase), immune factors (immune cells abundance and activity, lysozyme and complement activity, heat shock protein 70, high mobility group blot abundance, Toll-like receptor expression) and mortality rate were investigated. Moreover, the protein expression profile was investigated in the kidney, using a *without a priori* label free quantitative proteomics workflow. New pathways involved in immunity and other common metabolic pathways and affected by chronic hyperosmotic stressor in our study were highlighted and discussed.

In summary, hyperosmotic stressors were associated with an increase in plasma osmolality while gill NaK ATPase activity barely increased. Upon infection or not, salinity

enhanced tested innate immune factors. However, susceptibility to virulent bacterial strain of *Edwardsiella ictaluri* increased, especially in salt water. In kidney, salinity and infection exerted a synergic stress on the proteome's abundance. Salinity enhanced the general metabolic response (lipid metabolism, protein metabolism, energetics metabolism) as well as detoxification mecanisms (anti-oxidant, cytochrome P450). Numerous structural proteins (e.g. actin, collagen, spectrin) were differentially regulated depending on the osmotic pressure. In brackish water, several proteins involved in MAPKp38 signalling pathway, phagocytosis and T cells regulation were upregulated. In salt water, proteins implicated in inflammatory processes and the stress response were stimulated. However, the abundance of Toll-like Receptors (TLRs) transcripts, with the exception of TLR 5, decreased, especially in infected fish.

In conclusion, in striped catfish, the elevated internal body pressure caused by hyperosmotic stressors may induce inflammation whose intensity varies with stressor's intensity. The inflammatory response may unbalance the immune response due to pathogen attack (e.g. *Edwardsiella ictaluri*) infection and may interfere with the host-pathogen interaction, in the benefit of the pathogen when salinity exceeds 10 ppt. However, whether the fish controls inflammatory processes triggered by osmotic stimuli and whether inflammation interacts with the specific immune response remain to be investigated.

Acknowledgments

Je remercie mon promoteur Patrick Kestemont de m'avoir encadrée tout au long de ce travail et pour la confiance qu'il m'a accordée durant ces 4 années. Je le remercie également pour sa patience et son objectivité, ainsi que d'avoir su correctement doser à la fois un encadrement rigoureux et la liberté nécessaire à l'épanouissement du jeune chercheur dans son travail. Enfin, je vous remercie pour les quelques moments passés en dehors du laboratoire, en conférence dans le Maine soit à Can Tho au Vietnam, pour votre humour et votre énergie qui rendent ces voyages professionnels à la fois enrichissants et amusants.

I am deeply indebted to my examination committee, Peter Bossier, Eric Muraille and Lluis Tort for their insightful remarks and suggestions during the private defence. I thank Professor Tort for having travelled from Barcelona in order to participate at my thesis defence. I thank Prof. Muraille and Prof. Bossier for being part of my supervisory committee, for your time guidance across the phD program. I thank the Laboratory of Aquaculture and Artemia from the University of Ghent for their kindness and their generosity regarding Western Blot's antibodies. I thank Prof. Eric Depiereux, the chairman of this examination committee, for providing his time to facilitate the defence process. I would like also to express my gratitude to the Vietnamese team of the College of Aquaculture and Fisheries for their welcoming in Vietnam, the excellent guidance and the organization of the sampling campaigns we carried out in striped catfish ponds. Thanks are also due to the FNRS-FRIA, ARES-CDD and the project Deltaquasafe for financial support.

Je remercie ma co-promotrice Jessica Douxfils pour son encadrement tant au niveau du laboratoire qu'au niveau de la rédaction de ce travail, ainsi que pour nos nombreuses conversations en tant que jeunes mamans-poule passionnées par la recherche. Bonne continuation, ta présence au sein de notre équipe de recherche me manquera. Je remercie également énormément le professeur Robert Mandiki. Robert, tu n'avais aucune obligation d'encadrement me concernant et pourtant, tu as grandement contribué à l'élaboration de cette thèse malgré ton emploi du temps chargé et les nombreux étudiants et doctorants que tu encadres déjà. Merci également de m'avoir fait découvrir à travers nos passionnantes conversations ce magnifique pays qu'est la République Démocratique du Congo. Enfin, merci de m'avoir aidée à manipuler les Pangasius, malgré les nombreuses douches de vase que cela entraînaient.

Je remercie mes collègues pour leur participation à mes séminaires, aux journées de prélèvements et d'avoir su prendre soin des panga pendant mon congé de maternité ou mes deux longues périodes d'agonie après mon appendicite et ma triple fracture de la cheville (j'espère avoir la paix côté santé pour les dix prochaines années !!). Je remercie particulièrement Elodie pour son volontarisme et ses petits coups d'œil matinaux souvent bien utiles à l'état du circuit panga. Et bien sûr mon petit Sébastien (il faut décidément que j'arrête de te prendre pour mon étudiant !) pour sa complète dévotion envers son encadrante favorite ;). Adrien, je suis heureuse d'avoir trouvé un collègue de bureau avec lequel je peux me déconnecter du labo, qui a pu supporter mes fous rires, mes moments d'intenses bavardages, mon stress, mon côté renfermé et ronchon, ma parfois mauvaise humeur et d'avoir su me faire relativiser après mes journées de labo perdues (« Alors, tu as encore tout raté ? »). Enfin, merci aux deux hommes de l'ombre, André Evrard et Sascha Antipine, sans qui mes expériences auraient été impossibles. André, tu es le petit rayon de soleil souriant et plein d'humour du labo, je te souhaite bonne chance et paix pour cette retraite bien méritée.

Je remercie ma maman qui a souvent été à mon écoute et présente à mes côtés, ainsi que pour les libertés qu'elle m'a accordées dans ma vie notamment dans le choix de mes études.

Je dédie cette thèse à mes trois hommes sans qui je n'aurais jamais pu parcourir ce chemin. Papa, j'aurais souhaité que la vie nous accorde un peu plus de son temps si précieux. De là où tu es à présent, merci de m'avoir passionnée pour la biologie dès ma plus petite enfance et de m'avoir encadrée durant mes premières années à l'université, même si, soyons franc, tu étais un tantinet dépassé ! Cédric, merci pour ton aide à la maison comme au labo, ton calme, ton esprit posé et rationnel, tes déplacements à toute heure du jour ou de la nuit à mon travail ou à l'aéroport pour accueillir mes 2000 petits thailandais, et merci pour tes nombreux conseils avisés quant à mes publications, la rédaction de cette thèse ou mes présentations (non, je ne changerai pas mon titre !!). Nos mêmes passions pour la biologie et la recherche nous unissent et nous permettent de mieux nous comprendre, nous épanouir. Mon grinch, pour être honnête tu n'as absolument pas contribué scientifiquement à l'avancée de ce travail ; ton activité favorite étant d'appuyer sur toutes les touches de mon clavier, particulièrement quand je laissais mes feuilles de données ou mes publications en cours d'écriture ouvertes, soit de tester l'angle d'ouverture maximale de mon ordinateur laissé malencontreusement à portée de main. Néanmoins, il est crucial dans ce genre de travail de s'en détacher pour mieux savoir l'apprécier, d'en relativiser les échecs et d'apprendre la patience afin de ne pas finir complètement dévoré par le stress et la frustration à la fin d'une thèse. Tu m'as forcé de nombreuses fois à abandonner « l'interaction osmorégulation-immunité dans le protéome rénal» pour « la Panthère Rose », ce qui au final m'a permis de me vider la tête un instant et de redoubler d'énergie devant mon travail. Sans toi, je n'aurais jamais fini cette thèse l'esprit serein.

Table of content

Context of the study **13**

1. Striped catfish aquaculture 13
    1.1.Current state of world aquaculture insurance 13
    1.2.Aquaculture of striped catfish : Focus on the Mekong Delta 14
    1.3.Striped catfish 16

2. Global climate change: Implication in aquaculture 17
    2.1.Global view. 17
    2.2.Local prediction for the Mekong Delta: Focus on sea level rise. 18
    2.3.Consequences for striped catfish aquaculture. 19

3. Osmoregulation in fish 20
    3.1. General concepts 20
    3.2. Adaptive performance of striped catfish to salt stress 22

Objectives and outline of the thesis **25**

1. General objective. 25
2. Outline of the thesis. 25

I. The role of hyperosmotic stressor on the immune system and disease progression: An evolutionary perspective. **27**

1. Introduction 27
2. Osmotic implication in invertebrates. 28
    2.1. Overview of invertebrate immune system. 28
    2.2.Impact of hyperosmolarity on invertebrate immune system. 28
3. Osmotic implication in jawed fish 30
    3.1. Overview of fish immune system: A crossroad between innate and adaptive responses 30
    3.2.Impact of hyperosmolarity on fish immune system. 33
4. Osmotic implication in mammals 34
    4.1. Evolution of immune system in mammals 34
    4.2.Implication of hyperosmolarity on higher vertebrates immune system 35
5. The role of osmotic surveillance in inflammation: A link between osmotic response and immunity? 37
6. Discussion 41
7. Conclusions 42

II. Osmoregulatory and immunological status of the pond-raised striped catfish (*Pangasianodon hypophthalmus* S.) as affected by seasonal runoff and salinity changes in the Mekong Delta, Vietnam. **51**

1. Introduction 51
2. Materials and methods 53
    2.1.Field samplings 53
    2.2.Osmoregulatory parameters 55

2.3.Blood cell populations 55
2.4. Immune parameters 56
2.5.Statistical analysis 56
3. Results. 57
3.1.Osmoregulatory parameters 57
3.2.Blood cell populations 58
3.3. Immune parameters 59
4. Discussion 61
5. Conclusions 62

III. Chronic hyperosmotic stress interferes with immune homeostasis in striped catfish (*Pangasianodon hypophthalmus*, S.) and leads to excessive inflammatory response during bacterial infection. 67

1. Introduction 67
2. Material and method 69
2.1.Fish and in vivo stress experiment 69
2.2.Bacterial challenge 70
2.3.Osmoregulatory parameters 71
2.4.Hematology 71
2.5.Immune parameters 72
2.6.Statistical analysis 73
3. Results 73
3.1.Growth performance 73
3.2.Autopsy 74
3.3.Osmoregulatory parameters 74
3.4.Hematology 75
3.5.Immune parameters 76
3.6.Sensitivity to Edwardsiella 78
4. Discussion 79
5. Conclusions 81

IV. Low saline stress enhanced physiological and immune pathways in striped catfish *Pangasianodon hypophthalmus* (Sauvage). 85

1. Introduction 85
2. Material and method 86
2.1.Experimental design and statistical rationale 86
2.2.Bacterial challenge 87
2.3.Osmoregulatory parameters 88
2.4.Statistical analysis 88
2.5.Quantitative label-free proteomic analysis 88
3. Results 90
3.1.Osmoregulatory response of catfish to chronic saline stress 90
3.2.Proteomic study of catfish kidney proteome in response to chronic saline stress 90
3.3.Susceptibility to Enteric Septicaemia of Catfish 97
4. Discussion 97
5. Conclusions 101

V. Synergic stress in striped catfish (*Pangasianodon hypophthalmus*, S.) exposed to chronic salinity and bacterial infection: effects on kidney protein expression profile. 107

1. Introduction 107
2. Material and method 108
    - 2.1.Experimental design and statistical rationale 108
    - 2.2.Bacterial challenge 109
    - 2.3.Osmoregulatory parameters 110
    - 2.4.Statistical analysis 110
    - 2.5.Label free proteomics 110
3. Results 112
    - 3.1.Osmoregulatory capacities of striped catfish 112
    - 3.2.Analysis of kidney proteome 113
    - 3.3.Susceptibility to ESC 119
4. Discussion 119
5. Conclusions 123

VI. Chronic hyperosmotic stress inhibits renal Toll-Like Receptors expression in striped catfish (*Pangasianodon hypophthalmus*, Sauvage) exposed or not to bacterial infection 131

1. Introduction 131
2. Material and method 132
    - 2.1.Fish and in vivo stress experiment 132
    - 2.2.Bacterial challenge 133
    - 2.3.Quantitative PCR 133
    - 2.4.Statistical analysis 134
3. Results and discussion 134
4. Conclusions 137

VII. General discussion 141

1. Overview of the thesis 141
2. Acute and chronic stressors modulate the immune defences: Mechanisms of cross tolerance and synergy 142
3. What's link between osmoregulation and immunity? : discussion and Hypothesis 143
4. Which future for striped catfish farming? 146
5. Limitations of the thesis 146

VIII. General conclusions and perspectives 149

Context of the study

1. Striped catfish aquaculture

1.1 Current state of world aquaculture insurance

"*In a world where more than 800 million continue to suffer from chronic malnourishment and where the global population is expected to grow by another 2 billion to reach 9.6 billion people by 2050 – with a concentration in coastal urban area – we must need the huge challenge of feeding our planet while safeguarding its natural resources for future generation*". Jozé Graziano Da Silva, Director of the FAO.

Food fish (finfish, crustaceans and molluscs) production plays a crucial role in global food security and nutritional needs of people in developing and developed countries. In the last decade, global food fish consumption increased by 2.5 % per year, outpacing world human population growth (1.2%) (FAO, 2016). Global fish consumption per capita increased from 9.9 kg in the 1960s up to 20 kg in 2015 (FAO, 2016). The primary sector of aquaculture and fisheries engaged 56.6 million people in 2014, 84% of them living in Asia (FAO, 2016). The rapid population growth rate leads to an increasing demand for fish products that may not be covered by the fisheries sector. Indeed, since a production peak at 86.4 million tonnes in 1996, global fisheries exhibit a general decreasing trend to reach 93.4 million in 2014 (FAO 2016). The fraction of assessed stocks fished within sustainable levels decreased from 90% in 1974 to 68.6 % in 2013 (FAO, 2016). In 2013, 31.4% of fish stocks were overfished, 58.1% fully fished and 10.5 % underfished (FAO, 2016). Owing to the steady demand of fish products and the declining fisheries, politics and governments have turned to the support of the aquaculture sector (**Figure 1**).

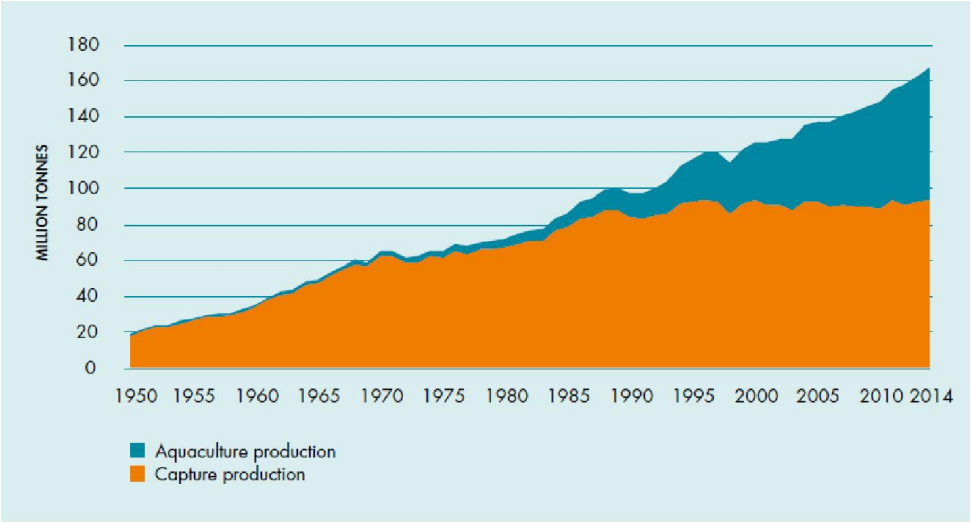


Figure 1: World capture fisheries and aquaculture production (FAO, 2016)

In 2014, world aquaculture production attained 101 million tonnes, including 49.9 million tonnes of finfish, 27.3 million tonnes of plants, 16.1 million tonnes of molluscs and 6.9 million tonnes of crustacea (FAO, 2016). In the recent years, developed countries such as United States of America, France, Spain or Japan decreased their aquaculture output due to competition with developing countries with lower production costs. The resulting fish food supply gap has been one of the main drivers encouraging aquaculture expansion in other countries with export-oriented specis. Asian aquaculture accounted for 84.3 % of world aquaculture production in 2014 (**Table 1**). China was the main producer of farmer fish with 27.2 million tonnes, followed by India (4.5 million tonnes), Indonesia (3.5 million tonnes) and Vietnam (2.7 million tonnes) (FAO, 2016).

Table 1: Aquaculture production by region (FAO, 2016).

	2000	**2005**	**2010**	**2012**	**2013**	**2014**
		(Thousands)				
Africa	4 175	4 430	5 027	5 885	6 009	5 674
Asia	39 646	43 926	49 345	49 040	47 662	47 730
Europe	779	705	662	647	305	413
Latin America and the Carribean	1 774	1 907	2 185	2 251	2 433	2 444
North America	346	329	324	323	325	325
Oceania	126	122	124	127	47	46
WORLD	**46 845**	**51 418**	**57 667**	**58 272**	**56 780**	**56 632**
OF WHICH, FISH FARMERS						
Africa	91	140	231	298	279	284
Asia	12 211	14 630	17 915	19 175	18 098	18 032
Europe	103	91	102	103	77	66
Latin America and the Carribean	214	239	248	269	350	356
North America	6	10	9	9	9	9
Oceania	5	5	5	6	5	6
WORLD	**12 632**	**15 115**	**18 512**	**18 861**	**18 818**	**18 753**

1.2 Aquaculture of striped catfish : Focus on the Mekong Delta

In several provinces of the Mekong River Delta, striped catfish has been produced since the 60s in floating cages, initially based on the capture of juveniles in the wild. Since the control of its whole life cycle in captivity, especially the artificial reproduction, the Vietnamese production of striped catfish increased tremendously and shifts from traditional cage farming to intensive production systems in ponds. In 2014, striped catfish production reached 1.2 millions of tons in Vietnam which represents almost 80 % of the total world production of this species (VASEP, 2015). The Mekong Delta includes 77 % of Vietnamese aquaculture production and is a region rich in aquatic resources with high potential for agricultural development (VASEP, 2015).

The striped catfish farming operations are rarely integrated and typically include hatchery, nursery and grow out sectors operating as independent entities and even with some degree of specialization in specific provinces. Hatcheries (87 in total) and nurseries (5535 in total) are concentrated in two provinces (An Giang and Dong Thap) whereas grow out operations (5442 in total) are dispersed in nine provinces. Hatchery production occurs especially through February to September and seedlings are reared in nurseries to a size of 4.5 cm for fry and 20 cm for fingerlings, when they are purchased by grow out farms (Phan et al., 2009). Most farms (76%) treat the seed before stocking and use to this purpose salt (78%) and antibiotics (32%) (Phan et al., 2009). Farmers treat ponds bottoms before filling with water and fish. The fallow period is highly variable, comprised between 2 and 45 days. During the fallow period, pond

treatment includes application of salt (11% of the farms), benzalkonium chloride (15% of the farms), agricultural limestone (27% of the farms) and chlorine (29% of the farms) (Phan et al., 2009). The amounts applied are highly variable and not regulated by specific guidelines. In addition, 82% of the farms perform sludge removal (Phan et al., 2009).

Catfish farms typically consist on average 4-m deep ponds (2.5 to 5.5 m), ranging in size from 0.035 – 0.1 ha for small scale farms (10 %) to more than 0.3 ha for large scale farms (60%) (Bosma et al., 2009; De Silva and Phuong, 2011). Each pond is surrounded with dykes in order to avoid the escape of fish during the flood season (Phan et al., 2009). Stocking density is high and averages 30 to 50 fish m^{-2} while yield reaches 70 to 850 t ha^{-1} (Phan et al., 2009; Ut et al., 2016). The water exchange rate range from 30% to 100 % replenishment, daily to once a week (Phan et al., 2009). Mean temperature averages 29.9±1°C and pH ranges from 6.7 to 7.8 but may rise up to 9 during algae blooms (Ut et al., 2016). Surface dissolved oxygen levels were constantly above 5 mg/L in the culture pond but is restricted to the top 1 m of the water column due to the absence of aeration (Lefevre et al., 2011a). Salinity measurements were never performed in striped catfish farms. However, salinity level up to 10 ppt were measured during the dry season in agricultural provinces (Nguyen et al., 2014). Fish are fed twice a day with commercial pellets containing 11% moisture, 20 to 30 % proteins, 6 to 8% fibre and 3 to 6 % lipids (Phan et al., 2009). Feeding rates range from 1 to 10 % body weight per day depending on fish body weight (Pan et al., 2009). Mekong Delta catfish farming operations are characterized by a rather high Feed Conversion Ratio (FCR) which averages 1.86±0.28 due to bad feed quality and composition (Bosma et al., 2009). The Specific Growth Rate (SGR) averages 1.1 % day^{-1} and fish reach their marketable size after 7 months (FAO 2013) (**Figure 2**).

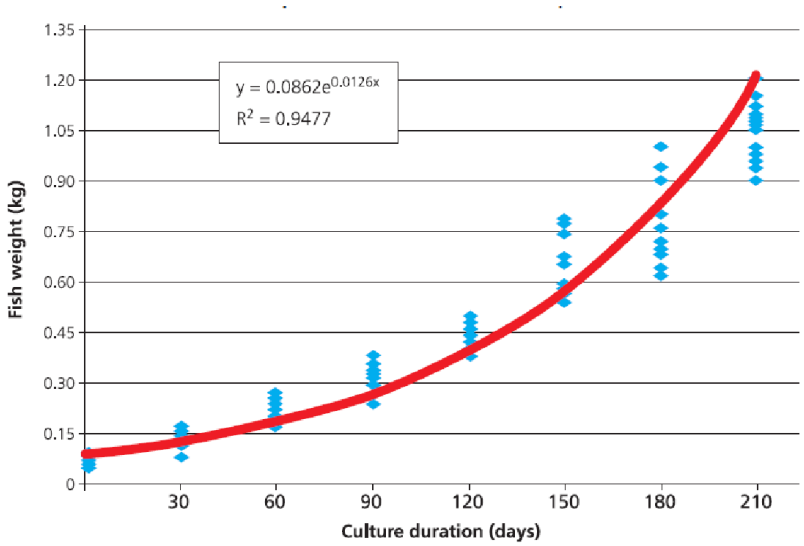


Figure 2: Variation of striped catfish body weight during 210 days (FAO, 2013).

Levels of mortality are highly variable from one farm to another. Following the first week of stocking, cumulative mortality reach on average 7% but may peak at 30 % (Phan et al., 2009). The main cause of mortalities in striped catfish farms is disease outbreaks. Farmers reported more than 15 symptoms and/or diseases. Among them Edwardsiellosis (98% of farms) and parasites (88% of the farms) are the most frequently occurring in striped catfish farms (Phan et al., 2009). Edwardsiellosis or Enteric Septicemia of Catfish (ESC) is by far the disease with the highest economic impact in striped catfish production (Crumlish et al., 2002). ESC is caused by the GRAM negative and intracellular bacteria *Edwardsiella ictaluri* (Hawke 1981). Higher

occurrence happens in June – July with the onset of the dry season and mortalities in infected farms may reach up to 90% of the fish stock (Phan et al., 2009).

1.3 Striped catfish

The striped catfish *Pangasionodon hypophthalmus* (Sauvage, 1878) (**Figure 2**) is a potamodromous freshwater fish endemic to the Mekong River Delta and the Chao Praya River in Thailand (Roberts & Vidthayanon 1991). Also known as shark catfish, tra catfish or Thai pangas, striped catfish may reach up to 1.30 m and 44 kg (Roberts & Vidthayanon 1991) (**Figure 3**).

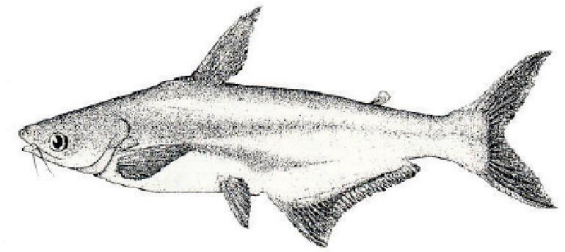


Figure 3: Striped catfish *Pangasianodon hypothalamus*, drawn by Vidthayanon. (Roberts & Vidthayanon 1991)

Few biological information is available for wild fish. In captivity, adults reach the sexual maturity around 2 to 4 years and are prolific spawners (up to 100 000 ova per fish kg) (Legendre et al., 2000). Like all Pangasiid species, striped catfish is a riverine migratory fish that makes several hundred kilometres between upstream refuges and downstream feeding habitats. At the end of the wet season (October to February), upstream migrations occur up to spawning refuges which consist on sandbanks and rapids interspaced with deep pools and deep rocky channels (FAO, 2010). Females lay their sticky eggs onto the root system of rheophilic tree species (Touch 2000). With the onset of the wet season (June to August), adults and larvae migrate downstream up to feeding habitats in the flood plains (Touch 2000).

Striped catfish is a highly tolerant species, which contributes to make this species one of the most economically important farmed fish in the world. First, this species is a facultative air-breather, with high capacity for aquatic and aerial respiration. At oxygen levels inferior to 8 kPa, striped catfish initiates air-breathing thanks to a modified swim bladder connected with the esophagus by a short pneumatic duct for gas exchange (Podkowa et al., 1998; Lefevre et al., 2011). In addition, striped catfish has well-developped gills and then differs from many other air-breathers where gills are reduced in order to limit oxygen loss when oxygenated blood from the swim bladder passes through the gills to reach the systemic circulation (Graham & Wegner, 2010). Indeed, the presence of a membranous flap of skin along the edge of the opercula allows diminishing the contact between gills and hypoxic water when opercula is closed during aerial respiration (Lefevre et al., 2011). Therefore, striped catfish may survive in oxygen levels as low as 2 kPa (Lefevre et al., 2011). In addition, this species is highly tolerant to nitrite with a lethal dose 50% which reaches 1.95 mM after 96h (Lefevre et al., 2011b). These particularities enable the fish to tolerate low dissolved oxygen level with poor quality water and high organic matter, allowing its culture under intensive pond conditions (up to 150 fingerlings / m^{-3}) (Rahman et al., 2006). Moreover, striped catfish are omnivorous and accept home-made feed formulated with agro and fishery by-products, pellets, trash fish as well as animal and human wastes (Sapkota et al., 2008).

2. Global climate change: Implication in aquaculture

2.1 Global view.

Climate change is projected to impact broadly across various ecosystems, economies and societies, increasing pressure on food supplies, including the sustainability of fisheries and aquaculture development. The effects of the climate change occur through gradual global warming effects and associated physical factors and take place in the framework of global socioeconomic pressure on environmental natural resources. The vulnerability of fisheries and aquaculture to climate change is summarized in **Table 2.**

Table 2: Vulnerability of fisheries and aquaculture systems to climate changes (FAO, 2014).

	Vulnerability										
	Overfishing	Drought	Variation in rainfall	Sea-level rise	Variation in sea surface temperature	Variation in currents	Acidification	Extreme weather events	Flooding	Changes in land use, damming	Volcanic eruptions, landslides, tsunamis
Lake Chad fisheries and farming		■	■								
Caribbean fisheries			■	■	■		■	■			■
Caribbean aquaculture							■	■	■		■
Mekong fisheries				■						■	
Mekong aquaculture									■	■	
Mekong rice				■					■	■	
Benguela fisheries	■					■					
Pacific fisheries					■		■				
Pacific aquaculture											
Pacific coastal habitats					■		■				
Latin America fisheries	■				■			■			
Latin America aquaculture							■	■		■	

Depending on the fish species and aquaculture localization, climate changes may result in positive and / or negative effects on aquaculture and fisheries. For example, in tropical Pacific freshwater aquacultures such as Nile tilapia (*Oreochromis niloticus*) farming operations, temperature increase should lead to faster growth rate, higher food conversion efficiency and farming will become possible in higher elevations (Bell et al., 2013). On the contrary, ocean acidification and higher sea surface temperature weaken shells and therefore should decrease the growth, survival and resistance to diseases in shellfish aquacultures such as pearl oysters (*Pinctada margaritifera*) (Bell et al., 2013). In addition, sea level rise will challenge number of coastal farming operations such as marine shrimps *Penaeus* sp. farmings (e.g. blue shrimps

(*Litopenaeus stylirostris*) in New Caledonia or tiger prawn (*Penaeus monodon*) in Vietnam) (Bell et al., 2013).

2.2 Local prediction for the Mekong Delta: Focus on sea level rise.

The Intergovernmental Panel on Climate Change (IPCC) (2013) estimated that more than 90% of the net energy increase of the climate system is stored in the oceans. According to 4 representative concentration pathways predicted by the IPCC (2013), global mean sea level rise by 2100 will range between 0.26 and 0.98 m (**Figure 4**). Ocean thermal expansion (30 to 55%) and glaciers melting (15 to 35%) are so far the dominant contributors to mean sea level rise. In the tropics, sea level rise induces seawater intrusion into deltaic areas such as the Bengali Delta or the Mekong Delta where considerable aquaculture production occurs.

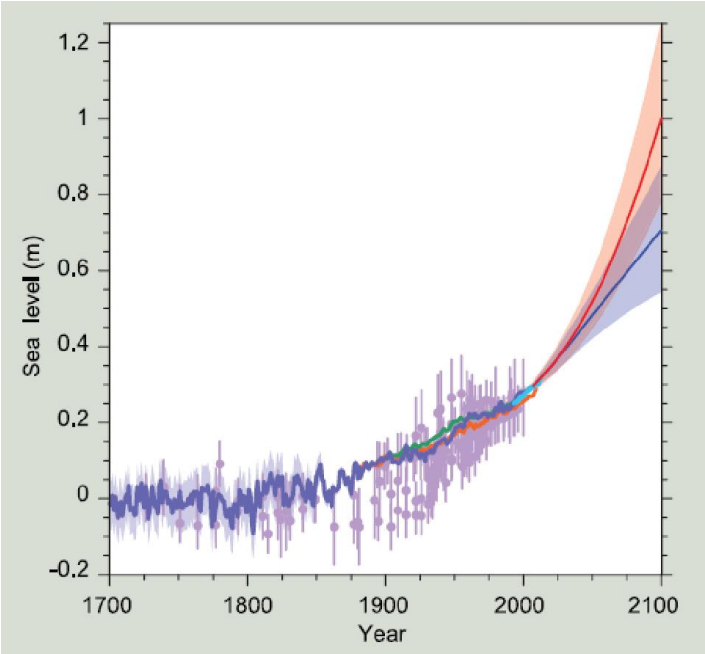


Figure 4: Compilation of tide gauge data (in green), altimeter data (in red), paleo sea level data (in purple), central estimates and likely ranges for projections of global sea level rise (IPCC 2013).

Several studies pointed out the effects of climate changes on the Mekong catchment (e.g. Wassman et al., 2004; Eastham et al., 2008; Nguyen et al., 2014). The most likely predictions have been assessed following 11 Global Changes Models from the fourth simulation of the IPPC by Eastham et al. (2008). In the south Basin of Mekong, Eastham et al.,(2008) predicted by 2030 an increase in mean temperature (+0.68°C-0.81°C) and precipitation (+51-100mm/year) compared to historical mean data (1951-2000), although projections may vary considerably for different catchments of the Basin. The disparity between the dry and wet season precipitations is thought to increase in all catchments, with higher precipitations during the wet season (May to October) and lower precipitations during the dry season. Mean annual runoff in lower catchments is thought to increase by 10 to 20%, mainly during the wet season (Eastham et al, 2008). In the dry season, runoff is likely to decrease by 6 mm (Eastham, et al., 2008). The most frequent impact of climate change will be on the elevated intensity and frequency of extreme events, particularly flood occurence (Eastham et al., 2008). In addition, sea level rise will exacerbate flood intensity and duration by reducing sediment's drainage efficiency (MRC, 2014). Nonetheless, these predictions do not take into account anthropogenic

effects such as storage hydropower or irrigation schemes that may exacerbate or moderate climate impact.

The FAO (2014) recognizes that the Mekong Delta is significantly vulnerable to sea-level rise and associated changes in salinity and flooding. During the dry season, decrease in water flow from 20 000 $m^3 s^{-1}$ to less than 5 000 $m^3 s^{-1}$ induces saltwater intrusion from the China Sea and the Gulf of Thailand into many coastal provinces of the Mekong Delta (MRC, 2010). During the dry season 2016, salinity infiltrates approximately 1.8 million ha of lands in the Delta, reaching more than 90 km inland (MRC, 2010; SIWRR, 2016) (**Figure 5**). In the tidal range, the mixing of seawater and freshwater induces modification in water circulation and helps to retain nutrients (MRC, 2010). On the coastal zone, salinity induces various environmental damages including loss of wetlands and population displacement, increased vulnerability to floods, accelerated erosion, salinization of groundwater aquifers and loss in agriculture lands, particularly rice crop (MRC, 2010; Smajgl et al, 2015). In the future, the climate changes will be responsible for extensive saltwater intrusion to the mouth of the river. Based on the Fourth and Fifth Assessment Reports of the IPPC, a sea level rise of 30 cm is predicted by 2050 for the coast of Southern Vietnam (Church et al., 2014).

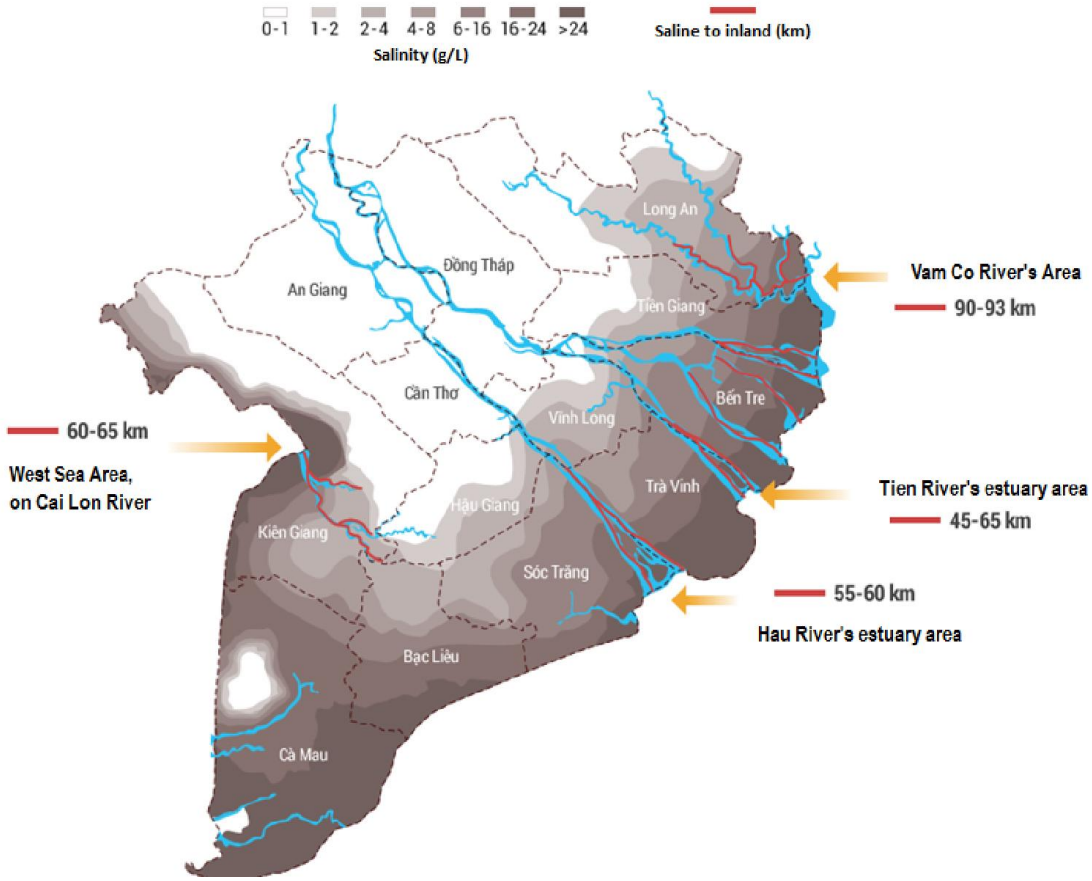


Figure 5: Salinity intrusion in the Mekong Delta in February 2016 (SIWRR, 2016).

2.3 Consequences for striped catfish aquaculture.

In the Mekong Delta, climate changes threatened the livelihoods of striped catfish farmers operating in the lower reaches of the two main branches of the river (Nguyen et al, 2015). The effect of climate change will increase additional cost of adaptation for catfish farmers and further reduce their profit margin. A study performed in 2008 reported that striped

catfish farms located downstream in the tidal range had lower yield (350 tonnes ha^{-1} $crop^{-1}$) compared to farms located upstream of the tidal range (450 tonnes ha^{-1} $crop^{-1}$) (Phan et al., 2009). In 2020, Kam et al. (2012) predicted that the projected benefits of coastal striped catfish aquacultures will be halved by climate changes compared to 2010, or a discounted net income of 4.7 billion VND ha^{-1} and showed that only farmers who can find innovative ways to substantially decrease their input cost will be able to survive in the long term.

The vulnerability of striped catfish farming to climate change has two aspects: exposure and sensitivity of striped catfish to climate changes and the perception of risk and possibilities for mitigation. (Nguyen et al., 2015). In the Mekong delta, 60% of fish farmers were aware of climate changes while about 50% considered adaptation measures (Nguyen et al., 2015). For all production systems including tiger prawns and striped catfish farms, about USD 172 million will be spent during the period 2010-2020 for dike upgrading and USD 18 million for increased costs in fuel and electricity due to climate changes (Kam et al., 2012). An alternative solution to the problem resides in the selection of salinity tolerant strain of striped catfish that might be speeded up by the genomic selection technology. The main advantage of this last solution relies on the minimal changes to perform in infrastructures and farming practises.

3. Osmoregulation in fish

3.1 General concepts

Osmoregulation gathers the cellular and systemic mechanisms that ensure the homeostasis of the internal osmotic pressure of an organism (~290 mosm L^{-1}). In the 1930s, August Krogh, Homer Smith and Ancel Keys demonstrated that freshwater teleosts were hyperosmotic to their environment whereas marine teleosts were hyposmotic to their environment. Their studies demonstrated that freshwater teleosts may actively extract ions from their environment via gill transport mechanisms while producing large volumes of hypotonic urine (**Figure 6**). On the contrary, marine teleosts actively excrete ions from gill epithelium while producing small volumes of isotonic urine (**Figure 6**). The gill is the primary sites of monovalent ionic transport whereas ingested divalent ions are excreted renally (Evans et al., 2009). The cellular sites for these ionic transports are chloride cells (also called mitochondria-rich cells), characterized by numerous mitochondria and an extensive tubular system (Mc Cormick, 2011). **Figure 7** shows chloride cells and their endocrine control in seawater and freshwater (Mc Cormick, 2011). In seawater, chloride cells are larger and contain deep apical crypts. Growth Hormone (and Insulin-like Growth Factor-1) and cortisol promote differentiation of seawater chloride cells and positively control epithelial transport capacity. In freshwater, chloride cells are smaller and contain microvilli. Prolactin promotes the formation of freshwater chloride cells whereas cortisol promotes acclimation by maintaining ion transporters and chloride cells. In addition, thyroid hormones may play a supportive role in osmotic adaptation, especially in salt excretory mechanisms during seawater acclimation through interaction with the GH/IGF axis (Mc Cormick, 2011). In migratory fish, growth hormone, prolactine and cortisol levels change in response to developmental and/or environmental cues such as temperature and photoperiod (Mc Cormick, 2011).

Salinity tolerance in organisms is determined by an effective coordination of various physiological systems, metabolic pathways and gene or protein networks. In general, consistent effects of salinity are difficult to identify and depend on the exposure time (acute versus chronic stress), the acclimation capacity of the organism and the intensity of the saline stressor. In fish, it has been estimated that 95% of the species are stenohaline and the remaining 5% are euryhaline (Mc Cormick, 2011). Euryhaline species have the capacity to tolerate large variation in their extracellular osmolarity and maintain osmotic homeostasis. On the other hand,

stenohaline regulators display lower capacity to maintain their osmotic homeostasis and thus tolerate a narrower range of salinity variation.

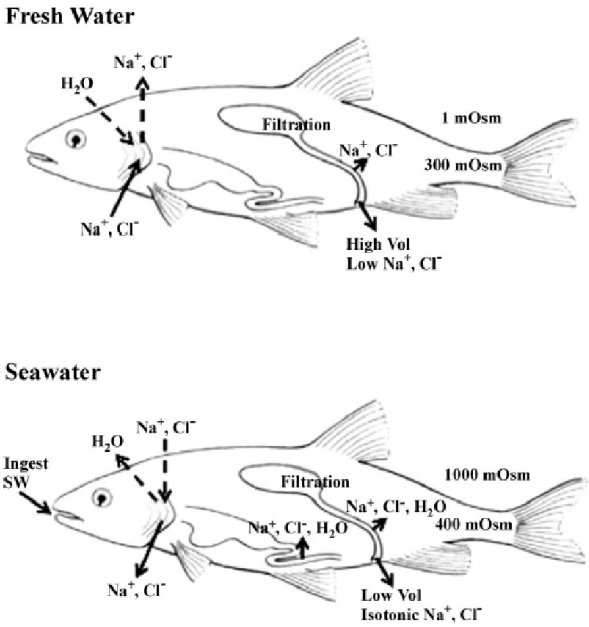


Figure 6: Teleost fish osmoregulation. Freshwater fish are hyperosmotic to their environment, so face passive gain of water and diffusional loss of ions across the permeable gill epithelium (dashed lines). Fish compensate by excretion of large volumes of dilute urine and active uptake of salt across the gill (solid lines). Seawater fish are hyposmotic to their environment, so face passive loss of water and diffusional gain of ions across permeable gill epithelium (dashed lines). Fish compensate by ingestion of seawater (intestinal reabsorption of NaCl and water), excretion of small volumes of blood-isotonic urine (after tubular reabsorption of NaCl and water) and active excretion of salt across the gill (solid lines). Evans et al., 2008.

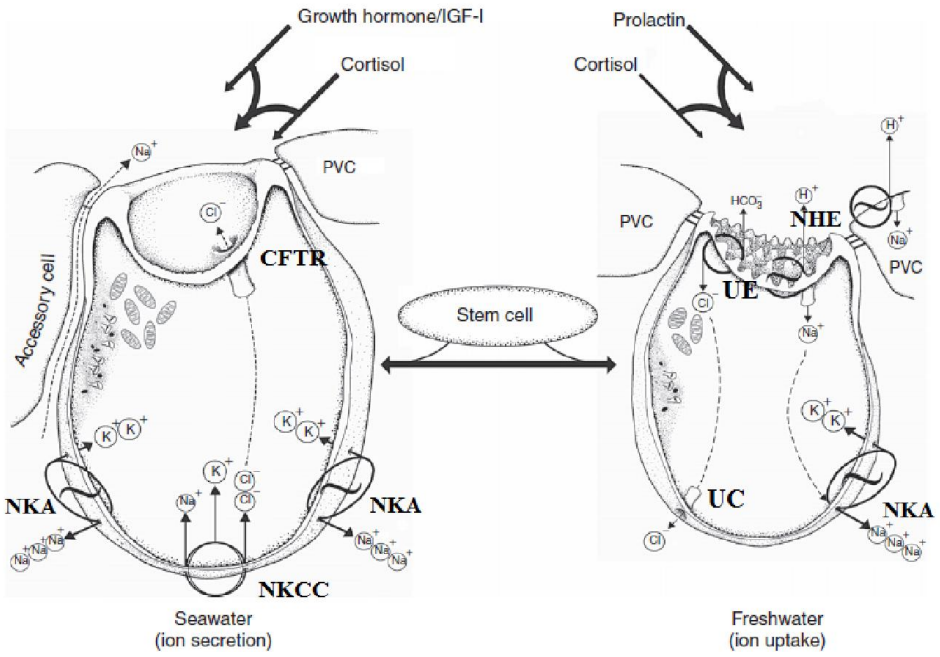


Figure 7: Chloride cells, ionic transporters and hormonal control in freshwater and seawater. PVC, pavement cells; NKA, Na^+K^+ATPase; NKCC, $Na^+K^+2Cl^-$ co transporter; CFTR, cystic fibrosis transmembrane; UE, undefined Cl^-HCO3^- exchanger; UC, undefined Cl^- channel; NHE: Na^+H^+exchanger. Mc Cormick, 2011.

3.2 Adaptive performance of striped catfish to salt stress

Few studies have been conducted in order to assess the adaptive performance of striped catfish in hypertonic media. Do et al. (2012) investigated the salinity tolerance of striped catfish eggs and larvae. Incubation of fertilized eggs in salinities up to 19 ppt suggested that embryos can develop and hatch up to salinities as high as 11 ppt. Survival threshold of striped catfish in salt water has not been determined yet but survival of juveniles (15-20g) during 56 days to salinities up to 18 ppt has been described (Phuc et al., 2014). Salinity levels up to 10 ppt did not impair neither fish weight gain not specific growth rate but elevated the feed conversion ratio (Phuc et al., 2014). Stress indicators (i.e. cortisol and glucose levels) increased after 6 h of salinity exposure (14 and 18 ppt), peaked 24 h after salinity exposure but decreased to basal levels after 14 days of acclimation (Phuc et al., 2014). Plasma osmotic pressure in striped catfish raised in freshwater averages 256 mosm L^{-1} and progressively increased after transfer in brackish water. At 18 ppt, corresponding to environmental osmotic pressure of 490 mosm L^{-1}, plasma osmolality peaks at 390 mosm L^{-1} (Phuc et al., 2014). Hypothetic changes in ionic transporters such as gill NaK ATPase in hypertonic media have not been investigated yet. To conclude, striped catfish does not appear to be efficient osmoregulator and survival to long term hyperosmotic stressor should limited due to the absence of efficient electrolyte excretion. The hypothetic impact of saline intrusion on striped catfish's immune defences and susceptibility to disease has not been investigated. Whether the decreasing yield in coastal farms is relied to decreasing susceptibility to pathogen, decreasing growth rates or both is unknown and will be deepen in this research.

References

Bell, J., Ganachaud, A., Gehrke, P., Griffith, S., Hobstay, A., Hoegh, O., Johnson, J., Leborgne, R., Lehodey, P., Lough, J., Matear, R., Pickering, T., Pratchett, M., Gupta, A., Senina, I., Waycott, M. Mixed responses of tropical Pacific fisheries and aquaculture to climate change. *Nat. Clim. Change*, 2013, *3*, 591-599.

Bosma, R.H., Hanh, C., Potting, J. Environmental impact assessment of the Pangasius sector in the Mekong delta. Agriculture, nature and food quality. Report from Wageningen university 2009, 1-58.

Church, J., Monselesan, D., White, N., Zhang, X., Platten, S. High resolution of climate change projections for Vietnam: Technical Reports. Eds Katzfey, J., McGregor, J., Ramasamy, S., 2014, *8*, 159-173.

Crumlish, M., Dung, T.T., Turnbull, J.F., Ngoc, N.T.N., Ferguson, H.W. Identification of Edwardsiella ictaluri from diseased freshwater fish *Pangasius hypophthalmus* (Sauvage) cultured in the Mekong Delta, Vietnam. *J. Fish Dis.,* 2002, *25,* 733-736.

Do, T.T.H., Tran, N.T. The effect of salinity on the embryonic development and osmoregulatory of the striped catfish (*Pangasianodon hypophthalmus*) larvae and fingerling stages. *Journal of Science (CTU*), 2012, 21, 29–37.

Eastham, J., Mpelasoka, F., Mainuddin, M., Ticehurst, C., Dyce, P., Hodgson, G., Ali R., Kirby, M. Mekong River Basin Water Resources Assessment, Impacts of Climate Change. CSIRO, Water for a Healthy Country National Research Flagship, 2008.

Evans, D.H. Teleost fish osmoregulation: What have we learned since August Krogh, Homer Smith, and Ancel Keys. *Am. J. Physiol. Regul. Integr. Comp. Physiol*., 2008, *295*, 704-713.

Food and Agriculture Organization of the United Nations. The state of world fisheries and aquaculture. Opportunities and challenges. FAO, Rome, 2014.

Food and Agriculture Organization of the United Nations. The state of world fisheries and aquaculture. Contributing to food security and nutrition for all. FAO, Sofia, 2016.

Ferdoushi, Z., Haque, F. Effect of different stocking ratio of Pangasid catfish (*Pangasius hypothalmus*) and silver carp (*Hypophthalmicthys molitrix*) on better water quality maintenance in catfish farming. *Pak. J. Biol. Sci*. 2006, *9(9*), 1732-1737.

Graham, J.B., Wegner, N.C., 2010. Breathing air in water and in air: the air-breathing fishes. In: Nilsson, G.E. (Ed.), Respiratory Physiology of Vertebrates: Life With and Without Oxygen. Cambridge University Press, pp. 174–221.

Handisyde, N.T., Ross, L.G., Badjeck, M.C., Allison, E.H. The effects of climate change on world aquaculture: a global perspective. Final Technical Report, DFID Aquaculture and fish genetics research program, Stirling, UK, 2006, 151 pp.

Hawke, J-P., McWhorther, A.C., Steigerwalt, A.G., Brenner, D.J. *Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicaemia of catfish. *Int. J. Syst. Bacteriol*. 1981, 31, 396-400.

Intergovernmental Panel on Climate Changes. The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, IPPC, 2013.

Kam, S., Badjecjk, M., Tee, L., Tran, N. Autonomous adaptation to climate change bu shrimp and catfish farmers in Vietnam's Mekong River Delta. Worldfish, 2012.

Lefevre, S., Huong, D.T.T., Ha, N.T.K., Wang, T., Phuong, N.T., Bayley, M. A telemetry study of swimming depth and oxygen level in a Pangasius pond in the Mekong Delta. *Aquaculture*, 2011a, *315,* 410-413.

Lefevre, S., Jensen, F., Huong, D.T.T., Wang, T., Phuong, N.T., Bayley, M. Effects of nitrite exposure on functional haemoglobin levels, bimodal respiration, and swimming performance of the facultative air breather fish *Pangasianodon hypophthalmus*. *Aquat. Toxicol*., 2011b, *104*, 86-93.

Legendre, M, Slembrouck, J., Subagjab, J., Kristanto, A. Ovulation rate, latency period and ova viability after Gn-RH of hCG-induced breeding in the Asian catfish *Pangasius hypothalamus. Aquat. Living Resour.,* 2000, *13,*145-151.

Mc Cormick S.D.The hormonal control of osmoregulation in teleost fish. In Farrell A.P., (eds) *Encyclopedia of fish physiology* : from genome to environment, *2*, 1466-1473, San Diego.

Mekong River Commission MRC (2010) State of the Basin Report 2010. Mekong River Commission, Vientiane, pp 123.

Nguyen, A., Dang, V. , Bosma, R. , Verreth, J. , Leemans, R. , De Silva, S.S. Simulated impacts of climate change on current farming locations of striped catfish (*Pangasianodon hypophthalmu*s, Sauvage) in the Mekong Delta, Vietnam. *Ambio*, 2014, *43(*8), 1509-1568.

Phuc, T., Huong, D., Mather, P., Hurwood, D. Experimental assessment of the effects of sublethal salinities on growth performance and stress in cultured tra catfish (*Pangasianodon hypophthalmus*). *Fish Physiol. Biochem*., 2014, *40*, 1839-1848.

Podkowa, D., Goniakowska-Witalinska, L., 1998. The structure of the airbladder of the catfish *Pangasius hypophthalmus.* Roberts and Vidthayanon 1991. Folia Biol. Krakow 46, 189–196 previously P. sutchi Fowler 1937.

Rahman, M., Islam, M., Halder, G., Tanaka, M. Cage culture of sutchi catfish *Pangasius sutchi*: effects of stocking density on growth, survival, yield, and farm profitability. *Aquaculture Res*., 2006, *37(1),* 33-39.

Roberts, T.R., Vidthayanon, C. Systematic revision of the Asian catfish family Pangasiidae, with biological observations and descriptions of three new species. *Proceedings of the Academy of Natural Sciences of Philadelphia*, 1991, 97-143.

Phan, L. T., Bui, T. M., Nguyen, T. T.: Gooley, G. J., Ingram, B. A., Nguyen, H. V., Phuong, N.T., De Silva, S. S. Current status of farming practices of striped catfish,

Pangasianodon hypophthalmus in the Mekong Delta, Vietnam. *Aquaculture*, 2009, *296*(3), 227-236.

Sapkota, A., Sapkota, A.R., Kucharski, M., Burke, J., Mc Kenzie, S., Walker, P., Lawrence, R. Aquaculture practise and potential human health risks: current knowledge and future priorities. *Environ. Int.,* 2008, *34(8*), 1215-1226.

SIWRR (The southern institute of water resources research) *http://www.tongcucthuyloi.gov.vn/, accessed on March 24, 2016.*

Smajgl, A., Toan, T.Q., Nhan, D.K., Ward, J., Trung, N.H., Tri, L.Q., Tri, V., Vu, P.Responding to rising sea levels in the Mekong Delta. *Nat. Clim. Change*, 2015, *5*, 167-174.

Touch, S.T. Life cycle of *Pangasianodon hypophthalmus* and the impact of catch and culture. Paper presented at the final meeting of the Catfish Asia Project in Bogor, Indonesia, 2000, 27 pp.

Ut, V.N., Giang, H.T., Phu, T.Q., Morales, J. and Phuong, N.T., 2016. Assessment of water quality in catfish (*Pangasianodon hypophthalmus*) production systems in the Mekong Delta. Can Tho University Journal of Science. Vol 3: 71-78.

VASEP-Vietnam Association of Seafood Exporters and Producers. Pangasius export statistics. VASEP, Vietnam, 2015.

Wassmann, R., Hien, N. X., Hoanh, C. T., Tuong, T. P. Sea level rise affecting the Vietnamese Mekong Delta: water elevation in the flood season and implications for rice production. *Climatic Change*, 2004, *66*(1-2), 89-107.

Objectives and outline of the thesis

1. General objective.

The general objective of this study was to better understand the mechanisms involved in the effects of hyperosmolarity on the immune system of the striped catfish *Pangasianodon hypophthalmus*.

First, we performed a field study in order to investigate the hypothetic impacts of seasonal chronic hyperosmotic stressor in striped catfish under aquaculture conditions. Then, we evaluated the stress and immune responses of striped catfish juveniles faced to gradual increases in salinity up to 10 ppt (low saline water) and 20 ppt (saline water) during 20 days in husbandry conditions and compared the results to values from freshwater fish to assess the impact of hyperosmolarity. We used common stress indicators and immune parameters but also a without *a priori* proteomic approach on the kidney in order to identify new immune pathways dysregulated by hypertonicity. Then, we decided to investigate the potential impact of salinity on the specific immune response. In this respect, we analysed the expression of Toll Like receptors using quantitative PCR.

We hypothesized that prolonged exposure to hyperosmotic conditions (10 and 20 ppt) induce chronic inflammation and therefore chronic activation of innate immunity. Then, we further hypothesized that following bacterial challenge, inflammation may result in excessive inflammatory responses and immune exhaustion, leading to tissue necrosis and serious diseases status.

2. Outline of the thesis.

Chapter 1 presents an introductive **review** which sums up the consequences of hyperosmotic stressors on the animal immune system and the initiation and progression of diseases following the evolutionary tree, in relation with the evolution of innate and adaptive immune defences. All taxa from invertebrates to mammalian species experiment local and/or systemic hyperosmotic stresses. In all taxa, hyperosmotic stressors have been linked to immune dysfunctions and local/systemic chronic/acute diseases. The molecular and cellular responses to hyperosmolarity tend to be conserved across the evolution, leading to many similarities between invertebrates, fish and mammals. However, body compartmentalization and evolution of the immune defences lead also to some divergences that are discussed in this review.

Chapter 2 draws up current key osmoregulatory (plasma osmolality, gill and kidney Na^+K^+ATPase) and innate immune parameters (blood cells, lysozyme activity, complement activity, respiratory burst) of striped catfish in fish farms. The field study was carried out during the dry season 2013 (March-April) and rainy season 2014 (July-August) in 12 fish farms of the lower (Tra Vinh Province) and higher (Can Tho Province) Mekong River Delta (Vietnam). The study investigated the variations of the osmoregulatory and immune factors depending on the geographical location, particularly the proximity to the River Mekong Estuary, and the seasonal environmental fluctuations, particularly the surface runoff.

The field study was complemented with an *in vivo* experiment. In **chapter 3**, striped catfish were submitted to three above mentioned salinity profiles (freshwater, low saline water, saline water) during 20 days, followed by infection with virulent bacteria, *Edwardsiella ictaluri*,

responsible for the enteric septicemia of catfish. The response of several indicators of the osmoregulatory (plasma osmolality, gill Na^+K^+ATPase) and immune (blood cells, lysozyme activity, complement activity, respiratory burst, Heat Shock Protein 70, High Mobility Group Blot-1) system was investigated before and following the inoculation of the bacteria. Moreover, the mortality rate after 10 days was assessed. The protein expression profile was investigated in the kidney of healthy and infected fish exposed to low saline water (**chapter 4**) and saline water (**chapter 5**) and compared to those in freshwater. In this purpose, a label free quantitative proteomics workflow was performed. The flow consisted in initial global profiling of relative peptide abundances (by LC/MS) followed by identification (by MS/MS). Differentially expressed proteins were clustered in functional categories and visualized in KEGG pathway maps. In low salinity-exposed fish, the responsiveness of immune proteins involved in small GTPases and Mitogen Activated Protein Kinase p38 signalling cascade, phagolysosome maturation and T-cells regulation is discussed. In high salinity-exposed fish, we highlighted several proteins involved in inflammatory process and stress pathways. Moreover, more common pathways such as those involved in energy metabolism, protein metabolism and processes, cell structure, detoxification and ion homeostasis were highlighted in both studies and debated. In the last experimental chapter (**chapter 6**), the expression of several Toll Like Receptors related to hypertonic stress in healthy and infected catfish in the whole kidney has been investigated.

Following the presentation of these scientific articles, a general discussion is proposed (**chapter 7**) is order to get a more general view of the results while trying to avoid redundancies with preceding chapters.

Eventually, some conclusions and perspectives are presented in **chapter 8**.

Chapter 1

The role of hyperosmotic stressor on the immune system and disease progression: An evolutionary perspective.

1. Introduction

Osmolarity is used to describe the number of solutes molecules per solution weight or volume. The total number of solutes molecules influences the osmotic pressure exerted by a given solution. In biological organisms, the presence of semi permeable membrane ensures cellular homeostasis by maintaining distinct extracellular and intracellular solute environments. An organism experiments a hyperosmotic stress when the extracellular fluid osmolarity is higher than that of the intracellular fluid.

Salinity tolerance in organisms is determined by an effective coordination of various physiological systems, metabolic pathways and gene or protein networks. In general, consistent effects of salinity are difficult to identify and depend on the exposure time (acute versus chronic stress), the acclimation capacity of the organism and the intensity of the saline stressor. For instance, euryhaline species have the capacity to tolerate large variation in their extracellular osmolarity and maintain osmotic homeostasis. On the other hand, stenohaline regulators display lower capacity to maintain their osmotic homeostasis and thus tolerate a narrower range of salinity variation.

Hyperosmolarity is known to be responsible for a number of deleterious effects such as cell shrinkage, protein decarbonylation, oxidative stress, DNA damage, cell arrest, cell and mitochondrial depolarization, promoting apoptosis (Brocker et al., 2012). In animal cells, increase in environmental osmolarity is counteracted by an acute stress response called "Regulatory Volume Increase", responsible for the activation of ion transporters and retention of monovalent ions. If the saline stressor persists, inorganic ions must be replaced by organic osmolytes in order to restore the electrochemical gradient across the cell and prevent macromolecular damages (Yancey, 2005; Alfieri et al., 2007). Osmolytes fall into four main chemical categories: urea, small carbohydrates (e.g. polyols, and sugars), aminoacids and derivatives (e.g. glycine, proline, taurine, ectoine) and methylamines (e.g. N-trimethylamine oxide or TMAO, glycine betaine) (Yancey, 2005; Holthauzen & Bolen, 2007). These categories are widespread in occurrence; for example glycine betaine is found in all kingdoms of life while taurine and TMAO are widespread in marine animals and mammals (Yancey, 2005). In general, organisms used complex mixtures of osmolytes which variety may result from unique properties of some osmolytes and /or may be simply due to different diets and metabolisms (Yancey, 2005). These osmolytes aim to control ion and water flux, stabilize macromolecules and counteract disruptors such as sulfides or oxygen radicals (Yancey, 2005; Brocker et al., 2012). Many osmolytes, such as heat shock proteins (HSP), also serve as chaperones and preserve the protein integrity (Yancey, 2005). Moreover, cells upregulate antioxidant mechanisms to regulate the overproduction of ROS (Reactive Oxygen Species) induced by the subsequent activation of the energetics metabolism. Eventually, cytoskeleton remodelling also takes place to increase cell rigidity and counteract cell shape changes induced by the excessive osmotic pressure (Di Ciano-Oliveira et al., 2006).

Hyperosmotic stresses have been linked to many immune dysfunctions and local or systemic diseases in every kingdom of life. Following the evolutionary tree, this review sums up the consequences of hyperosmotic stressors on the animal immune system and the initiation

and progression of diseases, in relation with the evolution of innate and adaptive immune defences. From the impact of salinity on the predation pressure in zooplankton communities, up to the correlation between hyperosmotic stressor and chronic systemic diseases such as diabetes and inflammatory bowel disease, this review aims to highlight similarities and divergences of immune system capacities in invertebrate, fish and mammal faced to hyperosmolarity condition.

2. Osmotic implication in invertebrates.

2.1 Overview of invertebrate immune system.

Invertebrate do not display the level of sophistication in immune responses compared to fish and higher vertebrates. Due to the absence of an effective adaptive immune system (i.e. Major HistoCompatibility (MHC) complex and T- and B-cell receptors), the efficiency of the invertebrate immune system relies mainly on the diversity of non-specific defences (Medzhitov et al., 2000). In invertebrates, the defensive mechanisms include three basic innate immune strategies: the physical barriers such as epithelial barrier and mucosal interfaces, the humoral immunity and the cellular immunity.

The immune system of invertebrate expresses a large panel of pattern recognition receptors (e.g. *Toll* receptors, C-type lectins, complement homologues, Lipopolysaccharide (LPS)- and glucan-binding proteins) and is highly responsive to the recognition of damage and pathogen-associated molecular patterns (DAMPs and PAMPs) (Ellis et al., 2011; Allam & Raftos 2015). In higher invertebrates, it appears that immune-competent cells express an extensively high molecular diversity (i.e. more than 18000 isoforms) of Immunoglobulin-domain-containing proteins generated through a mechanism of somatic diversification that reminds somatic recombination occurring in lymphocytes of higher vertebrates (Watson et al., 2005; Allam & Raftos 2015). In arthropods, for instance, diversification occurs by alternative splicing of the Dscam molecules while in molluscs, diversification occurs through a mechanism similar to somatic hypermutation which targets fibrinogen-related proteins (FREPs), lectin-like proteins able to form immune complex-like aggregates in the hemolymph. Invaders are detected by these humoral and hemocytes-bound recognition factors and trigger the production of cytokines that will mediate recruitment and activation of immune-competent cells (Allams & Raftos 2015).

Haemocytes are the backbone of the cellular defensive mechanisms and their diversity reflects the broad array of their functional capacities. In mollusks, three types of hemocytes have been detected among which small hemoblast-like cells, large and agranular phagocytic hyalinocytes and granulocytes (Aladaileh et al., 2007). One important feature of haemocytes is their capacity to perform chemotaxis and phagocytosis. Neutralization of ingested microbes are then facilitated by the activation of antimicrobial mechanisms such as release of oxygen and nitrogen oxide, production/release of antimicrobial peptides and hydrolytic enzymes such as lysozyme (Allams & Raftos 2015). Moreover, hemocytes can promote the initiation of proteolytic cascades leading to clotting and melanization through the phenoloxidase pathway (Gonzalez-Santoyo et al., 2011).

2.2 Impact of hyperosmolarity on invertebrate immune system.

Resistance to pathogen largely depends upon intrinsic (immune response) and extrinsic (environmental) factors that generally favour the pathogen or the host leading to disease or healing. The effect of environmental factors on these interactions is particularly important in osmoconformers such as marine invertebrates and stenohaline fish species. During the last

decades, the emergence of epidemic diseases in prawn and bivalve aquaculture farms during period of osmotic challenge has drawn attention to a potential impact of hyper- or hypoosmolarity on the immune system and disease resistance. In East Asia, the onset of the monsoon season correlates with outbreaks of white spot syndrome in penaeid shrimps *Penaeus monodon* (Son et al., 2010; Oseko et al., 2006). In the Chesapeake Bay (United States), the prevalence and intensity of infection with *Prochlorococcus marinus* in eastern oysters *Crassostrea virginica* have been positively correlated with salinity (Chu et al., 1993; Anderson et al., 1995). Along the Northeast coast of United States, the QPX (Quahog Parasite Unknown) disease epizootics, induced by a protistan parasite, are usually observed in hard clams *Mercenia mercenia* in field with high salinities (Perrigault et al., 2012).

In zooplanktonic communities, salinity acclimation may incur a loss of fitness in the context of local predation regimes. In calanoid copepod *Eurytemora affinis*, salinity interferes with the adaptive responses to oxidative stress and anti-oxidative activities (Cailleaud et al., 2007). In daphnid *Daphnia pulex*, adaptation to hypertonicity induces a dysregulation of more than 500 constitutive genes, including 64 genes involved in the plastic response to predation pressure, which corresponds to 11.3% of the total genes suspected to be involved in the kairomone response (Latta et al., 2012). On the other hand, daphnids display better resistance to saline stress in presence of fish kairomone due to the antagonistic interaction between salinity and predation pressure on lipid stores (Bezirci et al., 2012).

In many freshwater and seawater species of crustacean, long-term exposure to higher salinity stimulates the number of hemocytes (hyaline and granular subtypes), respiratory burst, phenoloxidase activity, lysozyme activity, antioxidant properties, phagocytic activity and pathogen clearance efficiency (Vargas et al., 1998; Le Moullac & Haffner 2000; Cheng et al., 2003; Yu et al., 2003). Similarly, in several species of bivalves, the acclimation during 6 or 7 days to hypersaline condition stimulates the number and activity of hemocytes (i.e. antioxidant defences, oxidative capacity, lysozyme activity, phenoloxidase activity and phagocytic activity) (Reid et al., 2003; Gagnaire et al, 2006; Matozzo et al., 2012; Jauzein et al., 2013; Carregosa et al., 2014). On the contrary, acute saline stressor rather leads to a depression of the immune system in crustaceans and molluscs. Thus, acute hyperosmotic stress decreased the number of hemocytes, phenoloxidase activity, antioxidant capacities, respiratory burst, phagocytic efficiency and pathogen clearance capacity in tiger prawn *Penaeus monodon,* Indian prawn *Fenneropenaeus indicus* and Taiwan abalone *Haliotis diversicolor* from 12 to 96 h after transfer (Cheng et al., 2004; Wang & Chen 2006; Joseph & Philip 2007; Vaseeharan et al., 2013). On the opposite, the white shrimp *Litopeneaus vannamei* showed an increase in the total hemocyte count, phenoloxidase and respiratory burst 12 to 72 h after acute transfer from 25 to 35 ppt (Wang & Chen 2005). Moreover, overexpression of two crustin antimicrobial peptides was observed 6 h after transfer in tiger prawn (Vatanavicharn et al., 2009). Eventually, acute salinity change from 30 to 35 ppt stimulated phagocytosis and antioxidant properties in the sea cucumber *Apostichopus japonica* (Wang et al., 2008). Increase in hemocytes' abundance and associated immune functions occurring after salinity stress is generally considered as a consequence of proliferation or movement of cells from tissues into haemolymph. On the contrary, decrease is more likely due to cell lysis or diapedesis.

Susceptibility is also affected by osmotic fluctuations and is generally correlated with the immune status of the organism. Rearing the white shrimp *L. vannamei* at high salinities increased the survival after injection with *Vibrio alginolyticus* and *Whispovirus* sp. (Lin et al., 2012). Similarly, the survival of Manila clams *Ruditapes phillipinarum* inoculated with *Vibrio tapetis* by injection to the pallial cavity increased in hypersaline media (Reid et al., 2003). On the contrary, acute hyperosmotic shock in Indian prawn increased the susceptibility after injection with Whispovirus from 6.7% to 76.7% (Vaseeharan et al., 2013). The same observation was made in tiger prawn faced to injected *Photobacterium damselae* and

Whispovirus sp. and in Taiwan abalone after injection of *Vibrio parahaemolyticus* into the pallial sinus (Cheng et al., 2004; Wang & Chen 2006; Joseph & Philip 2007).

However, salinity may also interfere with the host-pathogen interaction by affecting the growth and / or the virulence of the pathogen. For example, Perrigault et al., (2010; 2012) showed that the differences observed between salinity treatments in the immune response and susceptibility to disease in hard clams *Mercenia mercenia* infected with QPX (Quahog Parasite Unknow) were rather induced by the suppressor effect of hypotonicity on the parasite than on the immune status of the host itself. Indeed, infected hard clams acclimated during 4 months at 17 ppt showed higher hemocyte content and lower mortalities (less than 5%) than in clams elevated at 30 ppt (more than 15% of mortalities). At lower salinities, clams did not immunologically respond to QPX infection and the disease did not develop. The suppressive / stimulator effects of salinity have also been observed in other common pathogens affecting crustacean and mollusc health status. Exposure of *Vibrio harveyi* to low salinity levels (10 ppt and 15 ppt) for 12 h prior to challenge increased mortality during immersion challenge in tiger shrimp larvae (Prayitno & Latchford 1995). Addition of NaCl at 2.5% to 3.5% to tryptic soy broth significantly increased growth and virulence of *Vibrio parahaemolyticus* and *P. damselae* in Taiwan abalone and tiger prawn (Cheng et al., 2004; Wang & Cheng 2006). Therefore, it is difficult to isolate the effects of salinity on the host immune response without knowing the pathogen's salt tolerance, particularly in immersion challenge or osmoconformer species such as marine invertebrates.

3. Osmotic implication in jawed fish

3.1 Overview of fish immune system: A crossroad between innate and adaptive responses

The immune system of jawed fish is physiologically similar to that of higher vertebrates. Located at the crossroad in the evolutionary tree, jawed fish are the earliest class of vertebrates which possess both innate and adaptive immune defences although their adaptive response might be in some point less developed than that of higher vertebrates. The main difference between fish and higher vertebrates reside in the fact that fish are free-living organisms from early embryonic stage of life and therefore will primarily depend on their innate defences to survive (Uribe et al., 2011). Moreover, the fish immune response can be highly influenced by various external factors (e.g. temperature, salinity, water quality) in their own environment. Particularly, by their poikilotherm nature, temperature threshold limits immunocompetence and interferes with number of immune responses such as affinity maturation, T cell activation and innate immune parameters (Tort et al., 2003).

The innate system of fish is evolutionary highly conserved compared to invertebrates or higher vertebrates. As in other vertebrates, inflammation, orchestrated by cytokines, is the hallmark of the innate system which in the classical description entails swelling, chemotaxis of leukocytes, destruction and remodelling of tissues. Toll Like Receptors (TLRs) become the major receptors for the recognition of PAMPs and appear to be highly conserved through the vertebrate phyla (Lafyatis & Farina, 2012; Sunyer, 2012). Several intracellular pathways mediate the TLR effects, most importantly MyD88, which activates key intermediates such as NF-κB, mitogen-associated protein kinases (MAPK) and tat-associated kinases and eventually regulate the expression of inflammatory mediators such as cytokines (Lafyatis & Farina, 2012). However, some marked changes in the structure of the innate system can be seen through the phylogeny. For example, Natural Killer cells, type I interferon, eosinophils and basophils are unique of jawed vertebrates (Medzhitov, 2007). Moreover, programmed apoptosis by Natural Killer cells of virally infected cells is a viable defensive strategy of organisms which have

renewable tissues, which is not the case of invertebrates (Medzhitov, 2007). The complement system appears to be a central immune response in fish. Unlike mammals, fish possess several functionally isoforms of the C3, C4, C5 and factor B and the bacteriolytic and haemolytic activity of these C3 isoforms has been demonstrated to be higher compared to mammals (Sunyer & Tort, 1995; Sunyer & Lambris, 1997; Plouffe et al., 2008).

Prior to vertebrates, the immune response relied on non-lymphoid cells and serum molecules. The emergence of vertebrates marked the development of body compartments and cell organization. In fish, two parallel systems of adaptive immune strategies to generate antigen-specific lymphocyte receptors have evolved through rearrangement of leucine-rich repeats of Variable Lymphocytes Receptors (VLRs A, B, C) in jawless fish and VD(J) recombination of B-Cells Receptors (BCRs) and T-Cells Receptors (TCRs) in jawed fish (Pancer et al., 2004; Das et al., 2013).

Jawless fish lack recognizable immune organs, which consist on patches of thymus-like tissue called thymoid and located at the tips on gill filaments as well as gut-associated lymphoid-like tissue called typhlosone (Amemiya et al., 2007). Three distinct lymphocyte-like populations were highlighted and express specific cytokines and transcription factors (**Figure 1**). While VLRA and VLRC assembly on distinct VLRA+ and VLRC+ lymphocytes lineages takes place in the thymoid, VLRB expression on VLRB+ lymphocytes is rather observed in the typhlosole (Bajoghli et al., 2011; Holland et al., 2014). In jawed fish, lymphoid tissues consist on haematopoietic tissues mainly located in the head kidney (*Teleostei*), the spleen, the thymus and dispersed lymphoid aggregates located in the gut-associated lymphoid tissues (Tort et al., 2003). The head kidney assumes the haematopoietic function and is the principal immune organ responsible for antigen processing, antibodies production and immune memory through melanomacrophage centers (Tort et al., 2003). In hematopoietic tissues, T and B lymphocytes undergo VD(J) rearrangements of their antigen receptors mediated by recombination-activating genes (RAG) (Schatz et al., 1992). This process generates a diverse repertoire of receptors sufficient to recognize antigenic components of any potential pathogen or toxin (Schatz et al., 1992). The spleen functions as a major secondary lymphoid organ, with abundant IgM-producing B cells (Rauta et al., 2012). The thymus produces T lymphocytes, stimulates phagocytosis and antibodies' production by B cells (Tort et al., 2003).

The adaptive immune response of jawed vertebrates is mediated by specialized lymphoid cells including T lymphocytes, responsible for cell-mediated immunity and B lymphocytes, responsible for humoral immunity (Pancer et al., 2006). Conventional T cells form two functional groups of cytotoxic (killing) CD8+ T cells and helper (cytokine producing) CD4+ T cells. Cytotoxic T cells release molecules or activate Fas-apoptotic pathway to kill infected or cancerous cells through MHC class I pathway (Laing & Hansen, 2011). Helper T cells secrete cytokine and coordinate the activity of other immune cells through MHC class II pathway (Laing & Hansen, 2011). In fish, several studies based on sequencing and monoclonal antibodies suggest the presence of CD4+ helper, CD8+ cytotoxic T lymphocytes and Major Histocompatibility receptors (Grimholt et al., 2003; Uribe et al., 2011). However, T lymphocytes differentiation has not been demonstrated although fish contain many of the molecular orthologues associated with Th subsets (**Figure 1**). Moreover, teleost leukocytes are known to express critical co-stimulatory molecules including CD28, CD40, CD83 and a single copy of CD80/86 (similar to mammalian CD86 in structure and function) although their role in activating fish adaptive immune response is poorly understood (Zhang et al., 2009; Hansen et al., 2009; Gong et al., 2009; Zhu et al., 2014). Teleost B cells are located at the interface between innate and adaptive immunity, (Zhu et al., 2014). Indeed, they act as innate-like cells by showing potent phagocytic and strong microbicidal activities (Zhu et al., 2014). In addition, they show antigen-presenting ability to both soluble antigen and bacterial particles to CD4+ naive T cells by MHC class II and costimulatory signals (Zhu et al., 2014). This indicates that

they might be a major population of initiating Antigen Presenting Cells in priming naïve T cells, similarly to mammalian dendritic cells and macrophages. Furthermore, fish B cells express immunoglobulins on their surface and secrete antigen-specific antibodies in response to immune challenge. Although jawed fish do not perform Ig-class switch, at least three isotypes of immunoglobulins (IgM, IgD and IgT/Z) have been described in teleost and are expressed by distinct B cells lineages (Hansen et al., 2005; Danilova et al., 2005; Sunyer et al., 2012). Unspecific IgM is the main player in the systemic immune response while IgD and IgT/Z appear to be specialized in mucosal immunity. As in mammals, it is currently assumed that B cells with high affinity receptors are selected as memory B cells (Morrison & Nowak 2002). Indeed, fish are able to develop a memory response after a first exposure to a pathogen and induce a secondary response faster and of larger amplitude than the primary response (Whittington et al., 1994). Mechanism of antibody affinity maturation is not clear. It is currently thought that melanomacrophage centers may trap antigens for more than 3 months after immunization in order to perform hypermutation process in proliferating B cells (Magor, 2015).

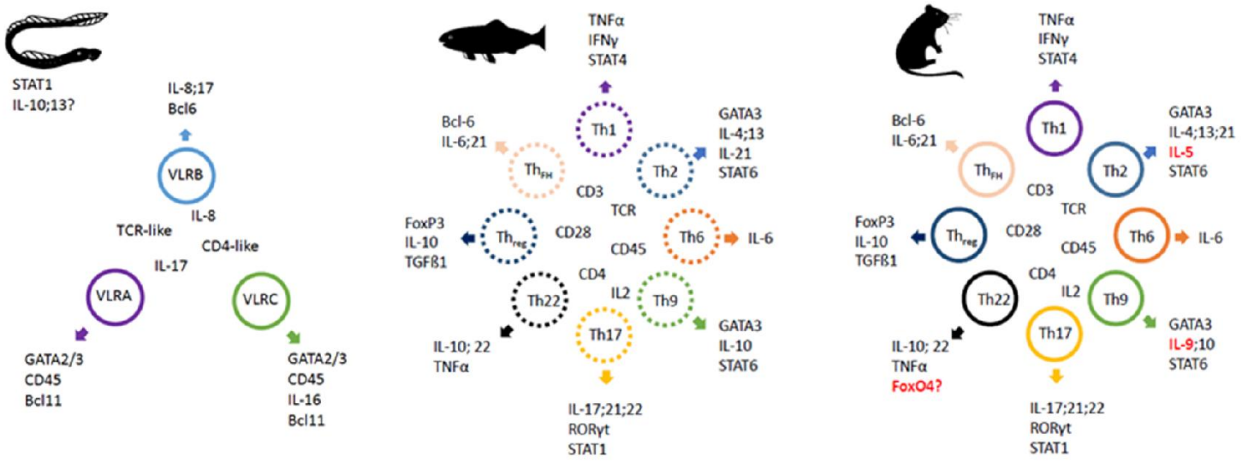


Figure 1: Signature cytokines and transcription factors expressed in distinct T-cells population, represented by different colors, in agnathan (left), teleost fish (middle) and mammals (right). In agnathans, the existence of 3 lymphocyte-like cell populations has been demonstrated and expressed distinct cytokines and transcription factors. Transcript of STAT1 and IL-10 and 13 receptors were discovered but not related to specific cell types. In teleost fish, many associated molecular orthologs have been identified although the existence of distinct T helper subsets is unknown (dashed circle). In mammals, T helper cells subsets produced or expressed specific signature cytokines and transcription factors. Molecular patterns specific to mammals were highlighted in red.

In euryhaline migratory fish, the immune system is strongly affected by shifting biotic (e.g. pathogen exposure, diet) and abiotic (e.g. dissolved oxygen, salinity, temperature) conditions encountered along the migration route. It is currently admitted that immune responses decline during migration. For example, in smolts of sockeye salmon (*Oncorhynchus nerka*) migrating in seawater habitats, genes related to cellular (e.g. MHC class I and II) and humoral (e.g. complement, interferon, immunoglobulin) responses were diminished (Evans et al., 2011). In addition, many genes involved in immune responses (e.g. IgE, lectins, MHC class II, Natural Killer cell enhancing factor, chemokine and cytokine-related genes) were downregulated in migratory silver European eels *Anguilla anguilla* (Kalujnaia et al., 2007). Similarly, antibacterial effector proteins and heavy chain immunoglobulin gene transcripts were

downregulated in migrating adult Chinook salmons *Oncorhynchus tshawytsha* in freshwater habitats (Dolan et al., 2016). Loss in immune functions during migration has been related to diminished energy reserves, hormonal changes and/or exposure to different pathogens (Evans et al., 2011; Dolan et al., 2016).

3.2 Impact of hyperosmolarity on fish immune system.

In stenohaline and euryhaline fish, chronic hyperosmotic stressor tends to stimulate the main indicators of the innate immune response. Increase in the abundance of leukocytes subtypes has been demonstrated in broad-nosed pipefish (*Syngnathus typhle*), and great sturgeon (*Huso huso*) acclimated to hypertonic environments (Zarejabad et al., 2010; Birrer et al., 2012). Gilthead seabream (*Sparus aurata*) acclimated during 100 days to brackish (12 ppt) and saline water (38 ppt) showed higher peroxidase and alternative complement activity in plasma compared to fish acclimated to low saline water (6 ppt) (Cuesta et al., 2005). In rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*), seawater-acclimated fish displayed higher lysozyme activity up to 90 days after transfer compared to freshwater-acclimated fish (Marc et al., 1995; Yada et al., 2001). Golden pompano (*Trachinotus ovatus)* reared for 30 days at 34 ppt displayed significantly higher superoxide dismutase activity than fish raised at 10 and 26 ppt (Ma et al., 2014). Likewise, the phagocytic index of brown trout, Mozambique tilapia (*Oreochromis mossambicus*) and broad-nosed pipefish leukocytes increased in fish exposed to hypertonicity (Marc et al., 1995; Jiang et al., 2008; Birrer et al., 2012). The effects of acute transfer in hypertonic environment have been relatively poorly investigated in fish. In euryhaline Mozambique tilapia, lysozyme activity, alternative complement activity and respiratory burst increased from 1 to 24 h after acute transfer from freshwater to 25 ppt saline water (Jiang et al., 2008). In Nile tilapia (*Oreochromis niloticus*), rapid salinity increases (0-5, 10 and 20 ppt) did not significantly affect neither the abundance of leukocytes nor the phagocytic index (Choi et al., 2013). However, these studies were biased by the fact that the Nile tilapia is a euryhaline species that can thrive in a wide range of salinity from freshwater close to full seawater and even, during a short period of time, hypersaline environments. Therefore, the impact of acute salinity changes on the fish immune system needs further investigation.

Several studies demonstrated the implication of inflammatory proteins, particularly molecular chaperones, during hyperosmotic stress. Gill proteome of Mozambique tilapia upregulated heat shock proteins and T-complex protein 1 members during short term exposure at 34 ppt, only HSP members during long-term exposure to 70 ppt and only HSP70 during long-term exposure to 90 ppt (Kültz et al., 2013). Moreover, two major gill cell types (chloride cells and pavement cells) of freshwater and seawater acclimated eels *Anguilla japonica* differentially expressed several immune proteins involved in the signalling of IL-6, IL-8, IL-9, HMGB-1 and iNOS (Lai et al., 2015). In addition, broad-nosed pipefish infected with *Vibrio* sp. lowered the expression of two key anti-inflammatory molecules (Granulocyte Colony Stimulating Factor and IL-10) after 3-days exposure to hyperosmotic conditions (6, 18 or 30 ppt) (Birrer et al., 2012). However, whether the osmotic stress serves as stimulus to trigger inflammatory response and subsequent immune modulation is still unknown.

The effects of hyperosmolarity on fish adaptive immunity have been poorly investigated, mainly because of the underestimation of adaptive immune capacities in fish in the past. A study on fish vaccination revealed that barramundi (*Lates calcarifier*) acclimated to seawater produced a higher adaptive mucosal antibody response than barramundi acclimated to freshwater (Delamare-Debouteville et al., 2006). In addition, gilthead sea bream (*Sparus aurata*) acclimated to hypersaline water (55 ppt) significantly increased total IgM levels compared to control (38 ppt) (Cuesta et al., 2005). In addition, the phosphorylation / activation

of the MAPK p38 signalling pathway in response to hyperosmotic stresses has been described in coho salmon, killifish and striped catfish (Kültz & Avila, 2001; Maryoung et al., 2015). MAPK p38 is a crucial mediator in many immunological processes including inflammation, T cells polarization, adhesion and chemotaxis (Ashwell, 2006). Eventually, the protein TAB2 was upregulated 4h after seawater transfer in the gill epithelium of Mozambique tilapia (Fiol et al., 2006). TAB2 is thought to be involved in B cell development as well as B cell activation in response to TLR stimulation leading to antigen-specific Ig responses (Ori et al., 2013).

Studies concerning the impact of hyperosmolarity on susceptibility to disease are scarce and used juvenile fish whose adaptive system is not completely formed. Hyperosmotic shocks in grouper fry (*Epinephelus sp.*) induced higher susceptibility after immersion with infectious pancreatic necrosis virus (Chou et al., 1999).

4. Osmotic implication in mammals

4.1 Evolution of immune system in mammals

The appearance of thermoregulation in warm-blooded vertebrates evolved with a higher degree of isolation from the surrounded environment and lower dependency on environmental conditions (Tort et al., 2003). The higher degree of self-regulation allowed higher vertebrates developing specific responses and reducing the number of variants of the innate molecules originating from most primitive organisms. Therefore, it is not surprisingly than the repertoire of several innate immune molecules (e.g. lectins, complement, natural killer receptors, NOD-like receptors) is less diverse in mammals than that of lower vertebrates (Sunyer & Lambris, 1997; Laing et al., 2008; Buchmann, 2014). However, the NOD-like receptors family expansion in mammals leads to a recent key regulatory mechanism of the inflammatory processes with the development of molecular platforms which coordinate the production and processing of inflammatory cytokines, called inflammasomes (Ogryzsko et al., 2014). In addition, innate immune cells are more easily identified on the basis of morphological and enzymatic features (eosinophils, basophils, neutrophils, phagocytes). The phagocyte function, taken essentially by macrophages in vertebrates (although other types of cells such as dendritic cells, fibroblast and B cells may also exhibit phagocytosis), shows increasing sophisticated level during the evolution. In higher vertebrates, phagocytes play a key role in the initiation and development of the adaptive immune response, which make then indispensable partners for T and B cells (Buchmann, 2014). Degraded material during the endocytic pathway can be processed by intracellular endopeptidases and presented to T cells through MHC class I or II process (Chen et al., 2008). In lower vertebrates, the mechanism of presentation of particulate antigen through the two aforementioned pathways remains unclear and whether phagocytosis is involved in adaptive processes remains to be investigated.

Higher vertebrates show a higher degree of sophistication in term of immune compartmentalization and specialization of the adaptive system. In mammals, the bone marrow becomes the haematopoietic organ, gut-associated lymphoid tissues get organized in Peyer patches and the lymphatic system evolves and forms lymph nodes, whose germinal centers become the major site of cell to cell interactions in mammals (Tort et al., 2003). In general, the adaptive immune system of higher vertebrates is characterized by a stronger memory response, a faster and stronger antibody response and higher affinity maturation (Tort et al., 2003; Sunyer et al., 2012). As an example, to generate significant antigen-specific IgM responses upon immunization, rainbow trout needs at least 3-4 weeks, in contrast to the few days required by mammals and antibody affinity increase was modest (3- to 10-fold affinity increase compared to 1000-fold increase in mammals) (Ye et al., 2010). In mammals, specific helper T cells subsets have been recognized through their activity and their unique signature of cell surface,

intracellular and secreted proteins (Th1, Th2, Th6, Th9, Th17, Th22, Tfh and Treg) (**Figure 1**). The main activities of Th subsets in mammals are summarized in **Table 1**. Proliferation of naive and memory T cells is regulated by TCR, cytokines and other surface receptors (Surth & Sprent 2008). Survival of naive T cells is sustained by IL-7 and TCR while memory T cells survival and expansion are regulated by IL-7 and IL-15 respectively (Surth & Sprent 2008). Whereas memory T cells maintained their phenotype during proliferation, naive T cells gradually evolved to a memory-like phenotype (Cho et al., 2000).

Table 1: Main functions of T helper cells subsets in mammals.

Th1	Mediate cellular immunity against intracellular infections
Th2	Mediate humoral immunity against extracellular pathogens Stimulate B cells proliferation and activation Drive antibodies mediated responses
Th6	Produce pro-inflammatory cytokine (IL-6) Involved in T cell regulation and differentiation
Th9	Promote inflammation
Th17	Promote inflammation to control extracellular bacterial infections Involved in autoimmune and chronic inflammatory disorders
Th22	Involved in epidermal repair
Follicular Th	Promote formation of long-lived antibody production in follicular B cells
Regulatory Th	Suppress the inflammatory response through cell to cell contact ($CD4^+CD25^+$ T cells) or anti-inflammatory cytokines TGF-β/IL-10 (Th3/ Tr1 T cells)

Regarding B cells, the mammalian B1 subset is thought to originate from a common ancestor and shares functional similarities with teleost B cells (Zhu et al., 2014). For instance, they show similar phagocytic and microbicidal capacities, produce IgM and share antigen presenting abilities to CD4+ naive T cells (Zhu et al., 2014). In contrast, mammalian B2 subsets are the main B cell population throughout the body, engaged in the activation of memory CD4+ T cells and producing specific high affinity antibodies (Strugnell & Wijburg 2010). While the TCR system remained similar throughout vertebrates, the BCR system (immunoglobulin receptors) has adopted specialized characteristics in different vertebrate phyla. Unlike teleost B cells, mammalian B cells are able to perform Ig class switching and the evolution resulted in increasing number and complexity of Ig isotypes, being maximal in mammals (IgM, IgA, IgD, IgE, IgG) (Tort et al., 2003; Zimmerman et al., 2009).

4.2 Implication of hyperosmolarity on higher vertebrates' immune system.

Terrestrial mammalian tissues such as kidney, cornea, intervertebral disks, liver and the gastrointestinal tract also experiment physiological fluctuations in osmolarity. In kidney, tubular epithelium is continuously exposed to high osmolality superior to 1000 mOsm (Burg et al., 2007). Also, the osmolality in tissue compartments can be affected by dietary salt intake or hydration state (Machnik et al., 2009).

In normal conditions, a growing body of evidences suggests that acute and chronic hyperosmotic stressors function as an inflammatory mediator, triggering pro-inflammatory cytokines release and inflammatory response (Brocker et al., 2012). In three distinct epithelial cell lines culture, hyperosmolarity over 300 mosm resulted in the secretion of pro-inflammatory cytokines IL-1ß, IL-6, IL-8 and TNFα (Schwartz et al., 2009). Similarly, cultured rabbit and human leukocytes showed increased production of IL-1, IL-2, IL-8, TNFα when faced to

hypertonic stressor (Junger et al., 1994; Shapiro & Dinarello 1997). In vivo, elevated plasma osmolarities (i.e. by hypertonic saline treatment or KCl injection) increased T cells proliferation and activity via MAPK p38 stimulation and ATP release (Loomis et al., 2003; Woerhle et al., 2010). In mouse, long-term oral administration of dextran sulfate sodium induced activation of p65 NF-κB, production of pro-inflammatory cytokines, swelling of lymph nodes and promoted colonic inflammation (Schwartz et al., 2008). Patients suffering from chronic renal failure and chronically exposed to solutions of 346–485 mOsm displayed chronic activation of T-cells (CD25 marker), B-cells (CD 23 marker) and macrophages (neopterin marker) as well as higher levels of IL-1, IL-6, IL-8, and TNFα (Caruana et al., 1992; Descamps-Latscha et al., 1995). In addition, in vitro long-term culture of macrophages at 600 mosm extended the half-life from 44 to 102 days, downregulated two pro-apoptotic proteins (p53 and Bax) and upregulated Bcl2, an anti-apoptotic protein, suggesting that chronic hyperosmolarity prevents apoptosis and allows elongation of macrophage half-life (Schwartz et al., 2009). Eventually, hypertonicity may restore the production of IL-2 from T cells in the presence of trauma patients or anti-inflammatory mediators (IL-4, IL-10, PGE2 and TGFß) (Loomis et al., 2001). On the contrary, the alternative, lectin and classical pathways of the complement cascade were dose-dependently attenuated with hypertonicity (Clark et al., 1992; Petersen et al., 2001). NLRP3 and NLRC4 inflammasomes play key roles in sensing hypertonicity of tissue microenvironment, inducing release in mature IL-1ß, and mediating inflammatory responses, neutrophil recruitment and activation of the adaptive Th17 pathway (Eddie & Medzhitov 2015).

The effects of hypertonicity on innate immune system highly depend on the timing of hyperosmotic stressor administration relative with immune cell activation. For example, if human neutrophils are exposed to hypertonic stressor before immune activation, osmotic shrinkage-associated conformational changes (e.g. peripherical actin proliferation) uncouple the receptor-mediated activation of the MAPK p38 and impair neutrophil's function (e.g. respiratory burst, adhesion molecules, degranulation, phagocytosis) (Murao et al., 2000; Junger et al., 2012). On the other hand, the further activation of the MAPK p38 by hyperosmolarity in stimulated cells may increase the neutrophil's inflammatory functions (Murao et al., 2000; Chen et al., 2006). The same mechanisms have been described in monocytes/macrophages. Indeed, hypertonic preconditioning in isolated macrophages decreased the LPS-induced activation of ERK1/2 and pro-inflammatory cytokines TNFα and IL-6 while increasing the production of anti-inflammatory IL-10 (Bode et al., 1998; Oreopoulos 2001; Cuschieiri et al., 2002). In addition, hypersaline infusions dextran just after hemorrhagic shock increased the production of anti-inflammatory cytokines (IL-10, IL-1ra) by the CD14+macrophage subset, and at the same time dramatically reduced TNFα production by the pro-inflammatory CD14+CD16+ macrophage subset, therefore restoring the inflammatory cytokine balance (Rizoli et al., 2006). Regarding Natural Killer cells, injection of hyperosmotic fluids did not change neither the number nor the activity up to 48h after surgery (Kolsen-Petersen et al., 2004).

In mammals, hypertonic fluids are largely used to ameliorate posttraumatic complications. In these clinical functions, massive tissue injury and ischemia, primes the innate immune system for excessive systemic inflammatory response syndrome (SIRS), leading to multiple organ dysfunction syndrome (MODS), the primary cause of death in patients who die in the intensive care unit. In these cases, excessive neutrophil activation contributes to tissue damages while suppression of the adaptive immune functions renders the patient susceptible to infections. In order to prevent these posttraumatic complication, hypertonic fluids, also called resuscitation fluids, have been shown to modulate postinjury hyperinflammation by attenuating neutrophil's inflammatory functions and enhancing T and B lymphocyte production (Ciesla et al., 2000; Kolsen-Petersen 2004). For example, after haemorrhagic shock, hypertonic saline infusions 4 ml kg^{-1} maintained phytohemagglutinin-induced T cell proliferation in spleen and skin whereas proliferation was suppressed by 40% in the isotonic lactated-Ringer group

(Coimbra et al., 1996). Similarly, preoperately patients infused with hypersaline (4 mg kg^{-1}) showed increased number in T cells and B cells after 24h (Kolsen-Petersen et al., 2004). In mouse, haemorrhage and resuscitation followed by puncture and ligation, reduced blood bacteremia as well as mortality after 72h from 77 to 14 % compared to the isotonic lactated-Ringer injected group (Coimbra et al., 1997). In models of rats with severe haemorrhagic shock, hypersaline infusions induced proliferation of lymphocytes and markedly increased the CD4+/CD8+ ratio of peripheral blood (Lu et al., 2007).

In human, a number of disorders such as cystic fibrosis, diabetes or Bowden disease have been related to local and / or systemic increase in extracellular fluid osmolarity and are tightly linked to inflammatory processes. Over recent years, it has been shown that local and peripherical hyperosmotic conditions might be a key factor behind the initiation and progression of many diseases. The pro-inflammatory cytokines related with hyperosmotic-related pathologies include TNF, IL-1ß, IL-6 and IL-8 (Brocker et al., 2012). Regarding eye disorders, in vitro and in vivo studies revealed that tear hyperosmolarity activates the MAPK p38 and NF-kappa B pathway, and upregulated the production of pro-inflammatory cytokines TNF, IL-1ß, IL-6 and IL-8 (Pan et al., 2011; Li et al., 2006). Patients exhibiting hypertonic hyperglycemia in serum were more than four times more vulnerable to diabetes than patients with hyperglycemia only (Stookey et al., 2004). Increasing osmolarity in the digestive tract has been correlated with Crohn's disease and colonic inflammation and cultured intestinal cells exposed to hypertonicity upregulated pro-inflammatory IL-1ß and IL-6 (Schilli 1982; Nemeth et al., 2002; Schwartz et al., 2008). Furthermore, salt-induced hypertension caused hyperplasia of lymph capillaries, production of nitric oxide by endothelium and plays key role in cardiovascular diseases such as coronary heart disease (Kannel et al., 1976). In periodontitis, osmotic pressure of gingiva was correlated with alveolar bone loss and gingival pro-inflammatory cytokines IL-1 and TNFα production (Lütfioğlu et al., 2011).

5. The role of osmotic surveillance in inflammation: A link between osmotic response and immunity?

In zebrafish, Enyedi et al. (2013) demonstrated the existence of an osmotic signalling pathway that monitors the tissue barrier integrity and serves as pro-inflammatory stimuli during tissue damage. Indeed, leukocyte recruitment to tail fin wounds was inhibited in zebrafish larvae incubated in isotonic medium compared to those incubated in hypotonic medium (similar to freshwater). This indicates that exposure of the zebrafish tail fin to hypotonicity is necessary to induce leukocytes recruitment. Moreover, exogenous DAMPS (ATP or epithelial cell extract) were not able to restore isotonic inhibition of leukocytes chemotaxis providing the evidence than leukocyte recruitment requires additional stimuli than the presence of DAMPS alone (Enyedi et al., 2013). On the contrary, injection of mRNA coding for cytosolic phospholipase A2 and bath immersion with eicosanoid arachidonic acid metabolites restored leukocytes recruitment, suggesting their implication in epithelial osmotic surveillance.

Similarly, mammalian tissues have developed sensory and signalling systems that monitor and regulate fluid osmolarity. The osmolarity of most physiological fluids is regulated within a narrow window of tolerance regarding osmotic fluctuations. For example, major parts of the upper digestive tract and potentially the lung are covered with hypotonic freshwater-like fluid (Joris et al., 1993). In addition, both liver and lymphoid organs like spleen and thymus are slightly hyperosmotic relative to serum in physiological conditions (Go et al., 2004). A small increase in extracellular osmolarity above physiological ranges may be perceived by osmosensors, induce the production of pro-inflammatory cytokines thereby trigerring immune responses (Brocker et al., 2012). In mammals, the differences in osmolarities between the external environment and internal body compartments (i.e. organs and interstitial fluids) might

be a signal evolved in freshwater organisms able to trigger inflammatory responses and potent immune modulation (Enyedi et al., 2013).

Whether hyperosmotic stressors (acute and chronic) might be perceived as a danger signal by disrupting constitutive local osmotic gradients is unknown. However, it is likely that increased internal body pressure unbalances the osmotic homeostasis of the organism, leading to the activation of the "osmotic surveillance signalling cascade", thereby trigerring inflammatory responses and immune cells activation. This hypothesis may at least partly explain the divergent immune responses between invertebrates and vertebrates faced to hyperosmotic stressors. Indeed, acute osmotic stressors lead to immunodepression in invertebrates whereas tend to enhance immune responses in vertebrates (i.e. fish and mammals). In addition, chronic hypertonicity enhances the immune responses in invertebrates and lead to better survival during bacterial and viral challenges. On the opposite, prolonged exposure to hypertonicity in vertebrates has been linked to excessive inflammatory responses, tissue damage and septicaemia. In invertebrates, the absence of body compartmentalization compromises the presence of local osmotic gradients. Therefore, the potent absence of osmotic surveillance mechanisms may hamper or limit the activation of immune defences during osmotic stress.

Table 2: Overview of the impact of acute and chronic hyperosmotic stressors on invertebrates, teleost fish and mammals.

	Stressor duration	Immune factor	↓/↑	Species	References
Invertebrates	Acute	Hemocyte abundance Hemocyte activity Respiratory burst Phenoloxidase activity Lysozyme activity Phagocytic index Antioxidant properties	↓↑ ↓↑ ↓↑ ↓ ↓ ↓	↓ **:** *Haliotis diversicolor; Penaeus monodon; Fenneropenaeus indicus.* ↑ *: Litopenaeus vannamei*	Cheng et al., 2004; Wang & Chen, 2005; Wang & Chen, 2006; Joseph & Philip, 2007; Vaseeharan et al., 2013.
	Chronic	Hemocyte abundance Hemocyte activity Respiratory burst Phenoloxidase activity Lysozyme activity Phagocytic index Antioxidant properties	↑ ↑ ↑ ↑ ↑ ↑	*Chamelea gallina ; Crassostrea gigas ; Macrocallista nimbosa*; *Mytilus galloprovincialis ; Penaeus californiensis ; Venerupis decussata* ; *Venerupis corrugata* ; *Venerupis philippinarum.*	Vargas et al., 1998; Le Moullac & Haffner, 2000 ; Cheng et al., 2003 ; Reid et al., 2003 ; Yu et al., 2003 ; Gagnaire et al., 2006 ; Matozzo et al., 2012 ; Jauzein et al., 2013 ; Carregosa et al., 2014.
Teleost fish	Acute	Immune cells abundance Immune cells activity Respiratory burst Phagocytic index Complement activity Lysozyme activity	— ↑ — ↑ ↑	↑ *: Oreochromis mossambicus* — *: Oreochromis niloticus*	Jiang et al., 2008 ; Choi et al., 2013.
	Chronic	Immune cells abundance Immune cells activity Respiratory burst Phagocytic index Antioxidant properties Complement activity Lysozyme activity Antibody specificity (mucus) Antibody specificity (serum) IgM abundance	↑ ↑ ↑ ↑ ↑ ↑ ↑ — ↑	*Huso huso ; Lates calcarifer; Oncorhynchus mykiss; Oreochromis mossambicus ; Pangasianodon hypophthalmus ; Salmo trutta ; Sparus aurata ; Syngnathus typhle ; Trachinotus ovatus .*	Marc et al., 1995 ; Yada et al., 2001 ; Cuesta et al., 2005 ; Delamare et al., 2006 ; Zarejabad et al., 2010 ; Birrer et al., 2012 ; Ma et al., 2014.

Mammals	Acute (normal conditions or after immune activation)	Immune cells abundance Immune cells activity Degranulation Phagocytic index Respiratory burst Adhesion molecules Secretion of inflammatory cytokines IL-1,2,6,8 ; TNFα Complement	↑ ↑ ↑ ↑ ↑ ↑ ↓	*Homo sapiens; Mus musculus. Oryctolagus cuniculus.*	Junger et al., 1994; Shapiro & Dinarello 1997; Murao et al., 2000 ; Chen et al., 2006 ; Schwartz et al., 2009.
	Acute (before immune stimulation)	Abundance of innate immune cells Abundance of adaptive immune cells Immune cells activity Degranulation Phagocytic index Respiratory burst Adhesion molecules Secretion of inflammatory cytokines IL-6;TNFα Secretion of anti-inflammatory cytokines IL-1ra; 10	↓ ↑ ↓ ↓ ↓ ↓ ↓ ↑	*Homo sapiens; Mus musculus; Rattus norvegicus.*	Coimbra et al., 1996 ; Bode et al., 1998 ; Murao et al., 2000 ; Oreopoulos et al., 2001 ; Cuschieiri et al., 2002 ; Rizoli et al., 2006 ; Lu et al., 2007 ; Junger et al., 2012
	Chronic	Immune cells abundance Immune cells activity T-cells marker (CD25) B-cells marker (CD23) Macrophage marker (neopterin) Macrophage half life Secretion of inflammatory cytokines IL-1,2,6,8 ; TNFα	↑ ↑ ↑ ↑ ↑ ↑	*Homo sapiens; Mus musculus; Orycttolagus cuniculus.*	Caruana et al., 1992 ; Junger et al., 1994 ; Descamps-Latscha et al., 1995 ; Schwartz et al., 2008 ; Schwartz et al., 2009.

6. Discussion

The evolution of the immune system tends to decrease the diversity of innate elements while increasing the complexity and diversity of adaptive defences. Indeed, whereas invertebrate immune defences mainly rely on the innate components (e.g. hemocytes and anti-microbial peptides), higher degree of compartmentalization in vertebrate allows the development of adaptive defences with a "self/non-self recognition system", the recognition of specific antigens and the presence of an immunological memory.

The molecular and cellular responses to a mechanical stress induced by hypertonicity tend to be conserved throughout the evolution, invertebrate and vertebrate defences showing similar responses depending on osmotic stressor's intensity and exposure time (acute versus chronic). The question is whether a long-term exposure may affect immune parameters at the same extent than short-term exposure. On one hand, it can be hypothesised that the impacts of hypertonicity may be more serious after long-term exposure of the animal to the stressful condition. On the other hand, a great ability of organisms to adapt their molecular, cellular and physiological parameters during long-term exposure in order to withstand to the stress cannot be excluded. In invertebrates, short-term hypertonicity (i.e. acute transfer of the organism in hyperosmotic environment) induces a depression of the innate defences, through inhibition of hemocytes' proliferation and related immune activities such as melanization or respiratory burst and may lead to higher susceptibility to disease. At the opposite, in normal conditions, short-term hypertonicity rather leads to immune enhancement in fish and mammals. However, in mammals, infusion of hypertonic fluids after trauma downregulated pro-inflammatory cytokines and innate inflammatory processes (e.g. leukocyte recruitment, adherence and activity) although suppression of innate components is bridged by enhancement of adaptive immune cells. Eventually, chronic exposure of invertebrates, fish and mammals to hypertosmotic conditions triggers inflammatory responses and positively modulates the immune response. In invertebrates, long-term exposure of crustaceans and molluscs has been correlated with better survival when faced to bacterial and viral diseases. On the contrary, long-term exposure of fish and mammals to hypertonic conditions has been related to lower survival during infectious disease, chronic inflammation and tissue damages.

In vertebrates, the evolution of body compartmentalization also allows the development of an "osmotic surveillance system", which monitors the tissue barrier integrity and triggers inflammatory responses when disruption of local osmotic gradients is perceived. This mechanisms might explain the higher responsiveness of the vertebrate immune system during hyperosmotic challenge compared to that of invertebrates.

Recent work has identified unexpected differences in resistance to abiotic stress among species. Species that thrive in fluctuating habitats such as the shallow subtidal, the intertidal or estuaries are pre-adapted to variations of abiotic variables. For instance, the immune system of *Chamelea gallina*, a sand-bottom species, was more vulnerable to a combination of abiotic changes than the intertidal-living species *Mytilus galloprovincialis* (Mattozo et al., 2012). Similarly, interspecific comparisons of the respiratory rates and metabolic changes between 10 species of bivalves and crustaceans faced to salinity changes lead to the conclusion that non-native species were more stress-resistant than native ones (Lenz et al., 2011).

Throughout evolution, proteins of the Rel/NF-kB/NFAT family and consequent activation of MAPK p38 cascade had critical roles in linking responses to osmoregulation and immunity. In *Drosophila*, the Rel/NFAT Family members regulate the expression of antimicrobial peptides against microbes (Imler & Hoffman 2000). In fish, MAPKp38 and associated proteins (eg G proteins, Cdc42) have been highlighted in several immune processes affected by hypertonicity. In mammals, Nuclear Factor of Activated T-cells 5 (NFAT5) controls the expression of genes involved in osmoprotection such as ion transport, production of organic

osmolytes and stress proteins (Neuhofer et al., 2010). Moreover, NFAT5 proteins target several immune genes implicated in the production of cytokines (TNFα and lymphotoxinß), activation of macrophages, T and B cell proliferation, B cell differentiation and IgG production (Neuhofer et al., 2010; Buxadé et al., 2012). NFAT5-null mice suffered from constitutive hypernatremia and severe immunodepression through lymphopenia, altered CD8 naive/memory homeostasis and inability to reject allogeneic tumors (Berga-Bolaños et al., 2010).

In the future, there is a need for studies that examine the consequences of salt stressors on the immune responses of further taxa. For instance, to our knowledge, nearly no information exist on the impact of elevated salinity on the immune system of amphibians, reptiles and birds. However, these taxa are particularly affected by salinization of their habitats via chemical runoff, secondary salinization and saline water intrusions associated with sea-level rise (Hart et al., 2003; Smith et al., 2007). Furthermore, whereas immune responses in migratory fish species have been discussed, the question of a potent energic cost of migration in other migratory species such as migratory birds should be also addressed. Therefore, this question should be expanded to other taxa.

7. Conclusion

This review highlights the impacts of hypertonicity on the immune defences and diseases, in relation with the evolution of innate and adaptive immune defences throughout the evolutionary tree. On one hand, the molecular and cellular mechanisms behind the osmotic response tend to be conserved throughout the evolution. On the other hand, immune responses following hyperosmotic stress may greatly depend on osmotic stressor's intensity, exposure time (chronic/acute) and the species' acclimation capacity (euryhaline/stenohaline). Whereas acute hyperosmotic stressors in normal conditions may result in immune depression in normal condition, chronic hyperosmotic stressor tends to induce inflammatory responses and the activation of innate cells. In the future, further questions should be addressed, such as the role of osmotic surveillance, the consequences for specific adaptive immunity and the consequences of salinization on amphibian and reptile's immune responses.

References

Aladaileh, S., Nair, S.V., Birch, D., Raftos, D.A. Sydney rock oyster (*Saccostrea glomerata*) hemocytes: morphology and function. *J. Invertebr. Pathol*. 2007, *96*, 48–63.

Alfieri, R., Petronini, P.G. Hyperosmotic stress response: comparison with other cellular stresses. *Pfülgers Archiv.* 2007, *454*, 173-185.

Allam, B., Raftos, D. Immune responses to infectious diseases in bivalves. *J. Invertebr. Pathol.* 2015, *131*:121-36.

Amemiya, C., Saha, N.R., Zapata, A. Evolution and development of immunological structures in the lamprey. *Curr Opin Immunol*., 2007*, 19(5),*535–541.

Anderson, R.S., Burreson, E.M., Paynter, K.T. Defense responses of hemocytes withdrawn from *Crassostrea virginica* Infected with *Perkinsus marinus. J. Invertebr. Pathol.* 1995, *66,* 82–89.

Ashwell, J.D. The many paths to p38 mitogen-activated protein kinase activation in the immune system. *Nat. Rev. Immunol*. 2006, *6*, 532-540.

Bajoghli, B., Gluo, P., Hirano, M., Strohmeier C., McCurley, N., Bockman, D., Schorpp, M., Cooper, M., Cooper, M., Boehm, T. A thymus candidate in lampreys. *Nature*, 2011, *470(7332),* 90-94.

Berga-Bolaños, R., Drews-Elger, K. , Aramburu, J. , López-Rodríguez, C. NFAT5 regulates T lymphocyte homeostasis and CD24-dependent T cell expansion under pathologic hypernatremia. *J. Immunol.* 2010, *185*, 6624-6635.

Berzirci, G., Akkas, S.B., Rinke, K., Yildirim, F., Kalaylioglu, Z., Severcan, F., Beklioglu, M. Impacts of salinity and fish-exuded kairomone on the survival and macromolecular profile of *Daphnia pulex*. *Ecotoxicology* 2012, *21,* 601-614.

Birrer, S.C., Reusch, T.B.H., Roth, O. Salinity changes impair pipefish immune defence. *Fish Shellfish Immunol.* 2012, *33,* 1238-1248.

Bode, J.G. , Peters, T. , Schliess, F. , Häussinger, D. Activation of mitogen-activated protein kinases and IL-6 release in response to lipopolysaccharides in Kupffer cells is modulated by anisoosmolarity. *J. Hepatol*. 1998, *28,* 795-802.

Brocker, C., Thompson, D., Vasiliou, V. The role of hyperosmotic stress in immunity and disease. *Biomol. Concepts* 2012, *3(4)*, 345-364.

Buchmann, K. Evolution of innate immunity: Clues from invertebrates via fish to mammals. *Front. Immunol.* 2014, *5*, 1-6.

Burg, M.B., Ferraris, J.D., Dmitrieva, N.I.. Cellular response to hyperosmotic stresses. *Physiol Rev*., 2007, *87*, 1441–74.

Buxadé, M., Lunazzi, G. , Minguillon, J. , Iborra, S. , Berga-Bolanos, R., Del Val, M. , Aramburu, J. , Lopez-Rodriguez, C. Gene expression induced by Toll-like receptors in macrophages requires the transcription factor NFAT5. *J. Exp. Med*. 2012, *209(2)*, 379-393.

Cailleaud, K., Maillet, G., Budzinski, H., Souissi, S., Forget-Leray, J. Effects of salinity and temperature on the expression of enzymatic biomarkers in *Eurytemora affinis* (Calanoida, Copepoda). *Comp. Biochem. Physiol. A*, 2007, *147*(4), 841-849.

Carregosa, V., Velez, C. , Soares A.M., Figueira, E., Freitas, R. Physiological and biochemical responses of three Veneridae clams exposed to salinity changes. *Comp. Biochem. Physiol*. 2014, 177, 1-9.

Caruana, R.J., Leffell, M.S., Lobel, S.A., Campbell, H.T., Cheek, P.L.. Chronic T-lymphocyte activation in chronic renal failure: a study of hemodialysis, CAPD and pre-dialysis patients. *Int. J. Artif. Organs* 1992, *15*, 93–98

Chen Y., Hashiguchi , N., Yip, L, Junger, W.G. Hypertonic saline enhances neutrophil elastase release through activation of P2 and A3 receptors. *Am. J. Physiol*. 2006, *290(4)*, C1051-C1059.

Chen, X., Jensen, P.E. The role of B lymphocytes as antigenpresenting cells. Arch. Immunol. *Ther. Exp*. 2008, 56, 77–83.

Cheng, W., Juang, F.M., Chen, J.C. The immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus* at different salinity levels. *Fish Shellfish Immunol*. 2004, *16*, 295-306.

Cho, B. K., Rao, V. P., Ge, Q., Eisen, H.N., Chen, J. Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. *J. Exp. Med.* 2000, *192*, 549-556.

Choi, K., Cope, W.G., Harms, C.A., Law J.M. Rapid decreases in salinity, but not increases, lead to immune dysregulation in Nile tilapia, *Oreochromis niloticus*. *J. Fish Dis*. 2013, *36(4),* 389-399.

Chou, H.Y., Peng, T.Y., Chang, S.J., Hsu, Y.L., Wu, J.L. Effect of heavy metal stressors and salinity shock on the susceptibility of grouper (*Epinephelus* sp.) to infectious pancreatic necrosis virus. *Virus Res.* 1999, *63,* 121-129.

Chu, F., La Peyre, J.F. , Burreson, C.S. *Perkinsus marinus* infection and potential defense-related activities in eastern oysters, *Crassostrea virginica*: Salinity effects. *J. Invertebr. Pathol.*, 1995, *62*, 226–232.

Ciesla, D.J., Moore, E.E. , Zallen, G. , Biffl, W.L. , Silliman, C.C. Hypertonic saline attenuation of polymorphonuclear neutrophil cytotoxicity: timing is everything. *J. Trauma*, 2000, *48*, 388–395.

Clark, E., Nath, K.A., Hostetter, T., Hostetter, M. Hyperosmolality impairs ammonia-mediated inflammation. Implications for the renal medulla. *Am. J. Physiol*., 1992, *263*, R148-155.

Coimbra, R., Junger W.G. , Hoyt, D.B. , Liu, F.C. , Loomis, W.H., Evers, M.F. Hypertonic saline resuscitation restores haemorrhage-induced immunosuppression by decrease prostaglandin E2 and interleukin 4 production. *J. Surg. Res.*, 1996, *64*, 203-239.

Coimbra, R., Hoyt, D.B. , Junger, W.G. , Andre, N. , Wolf, M. , Loomis, W., Evers, M.F. Hypertonic saline resuscitation decreases susceptibility to sepsis after hemorrhagic shock. *J. Trauma*, 1997, *42*, 602-606.

Cuschieiri, J., Gourlay, D., Garcia, I., Jelacic, S. , Maier, R.V. Hypertonic preconditioning inhibits macrophage responsiveness to endotoxin. *J. Immunol*., 2002, *168,* 1389-1396.

Cuesta, A., Laiz-Carrion, R., Martin del Rio, M., Meseguer, J., Mancera, J., Esteban, M. The salinity influences humoral immune parameters of gilthead seabream (*Sparus aurata*). *Fish Shellfish Immun.,* 2005, *18*, 255-261.

Danilova, N., Bussmann, J., Jekosch, K., Steiner, L.A. The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. *Nat Immunol*., 2005, *6(3),* 295–302.

Das, S., Hirano, M., Aghaallaei, N., Bajoghli, B., Baehm, T., Cooper, M. Organization of lamprey variable lymphocyte receptor C locus and repertoire development. *PNAS*, 2013, *110*, 6043-6048.

Delamare-Deboutteville, J., Wood, D., Barnes, A.C. Response and function of cutaneous mucosal and serum antibodies in barramundi (*Lates calcarifer*) acclimated in seawater and freshwater. *Fish Shellfish Immunol*., 2006, *21,* 92-101.

Descamps-Latscha, B. , Herbelin, A. , Nguyen, A.T. , Roux-Lombard, P. , Zingraff, J. *et al.,* Balance between IL-1β, TNF-α, and their specific inhibitors in chronic renal failure and maintenance dialysis. Relationships with activation markers of T cells, B cells, and monocytes. *J. Immunol*., 1995, *154*,, 882–892

Di Ciano-Oliveira, C, Thirone, A.C.P., Szászi, K., Kapus, A. Osmotic stress and the cytoskeleton: the R(h)ole of Rho GTPases. *Acta Physiol*., 2006, *187*, 257–272.

Dolan, B., Fisher, K., Colvin, M., Benda, S., Peterson, J., Kent, M., Schreck, C. Innate and adaptive immune responses in migrating spring run adult chinook salmon Oncorhynchus tschawytscha. *Fish Shellfish Immunol*., 2016, *48*, 136-144.

Eddie, W.K., Medzhitov, R. Macrophages monitor tissue osmolarity and induce inflammatory response through NLRP3 and NLRC4 inflammasome activation. *Nat Commun*., 2015, *6,* 6931.

Ellis, R.P., Parry, H., Spicer, J.I., Hutchinson, T.H., Pipe, R.K., Widdicombe, S. Immunological function in marine invertebrates: responses to environmental perturbation. *Fish Shellfish Immunol*. 2011, *30*, 1209–1222.

Enyedi, B., Kala, S., Nikolich-Zugich, T., Niethammer, P. Tissue damage detection by osmotic surveillance. *Nat. Cell. Biol.,* 2013, *15*, 1123-1132.

Evans, T., Hammill, E., Kaukinen, K., Schultze, A., Patterson, D., English, K., Curtis, J., Miller, K. Transcriptomics of environmental acclimatization and survival in wild Pacific sokeye salmon *Oncorhynchus nerka* during spawning migration*. Mol Ecol,* 2011, *20*, 4472-4489.

Fiol, D.F., Chan, S.Y., Kültz, D. Identification and pathway analysis of immediate hyperosmotic stress responsive molecular mechanisms in tilapia (*Oreochromis mossambicus*) gill. *Comp. Bioch. Physiol. D1*, 2006, 244-356.

Gagnaire, B., Frouin, E., Moreau, K., Thomas, H. , Renault, T. Effects of temperature and salinity on haemocyte activities on the Pacific oyster *Crassostrea gigas* (Thunberg). *Fish Shellfish Immunol.*, 2006, *20*, 536-547.

Gong, Y.F., Xiang, L.X., Shao, J.Z. CD154-CD40 interactions are essential for thymus-dependent antibody production in zebrafish: insights into the origin of costimulatory pathway in helper T cell-regulated adaptive immunity in early vertebrates. *J. Immunol*., 2009, *182(12)*, 7749–7762.

Go, W.Y., Liu, X., Roti, M.A., Liu, F., Ho, S.N. NFAT5/TonEBP mutant mice define osmotic stress as a critical feature of the lymphoid microenvironment. *Proc. Natl. Acad. Sci. USA*, 2004, *20*, 101(29), 10673-10678.

Gonzalez-Santoyo, I., Cordoba-Aguilar, A. Phenoloxidase: a key component of the insect immune system. *Entomol. Exp. Appl.*, 2011, *142*, 1-16.

Grimholt, U., Larsen, S. , Nordmo, R., Midtlying, P., Kjoeglum, S., Storset, A., Saebø, S., Stet, R.J. MHC polymorphism and disease resistance in Atlantic salmon (Salmo salar), facing pathogens with single expressed major histocompatibility class I and class II loci. *Immunogenetics*, 2003, *55*, 210-219.

Hansen, J.D., Landis, E.D., Phillips, R.B. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proc. Natl. Acad. Sci. USA*. 2005, *102(19),* 6919–6924.

Hansen, J.D., Du Pasquier, L., Lefranc, M.P., Lopez, V., Benmansour, A., Boudinot, P. The B7 family of immunoregulatory receptors: a comparative and evolutionary perspective. *Mol. Immunol*., 2009, *46(3),* 457–472.

Hart, B. T., Lake, P. S., Webb, J. A., & Grace, M. R. Ecological risk to aquatic systems from salinity increases. *Australian Journal of Botany*, 2003, *51*(6), 689-702.

Holland, S. J., Gao, M., Hirano, M., Iyer, L. M., Luo, M., Schorpp, M., Cooper, M.D., Aravind, L., Mariuzza, R.A., Boehm, T. Selection of the lamprey VLRC antigen receptor repertoire. *Procl. Nat. Acad. Sci.*, 2014, *111*(41), 14834-14839.

Holthauzen, L.M.F., Bolen, D.W. Mixed osmolytes: the degree to which one osmolyte affects the protein stabilizing ability of another. *Prot. Sci.,* 2007, *16,* 293-8.

Imler, J.M., Hoffman, J.A. Signaling mechanisms in the antimicrobial host defense of Drosophila. *Curr. Opinion Microbiol*., 2000, *3(1)*, 16-22.

Jauzein, C., Donaghy, L., Volety, A.K. Flow cytometric characterization of hemocytes of the sunray venus clam *Macrocallista nimbosa* and influence of salinity variation. *Fish Shellfish Immunol*., 2013, *35,* 716-724.

Jiang, I-F., Khumar, V-B., Lee, D-N., Weng, C-F. Acute osmotic stress affects tilapia (*Oreochromis mossambicus*) innate immune responses. *Fish Shellfish Immunol*., 2008, *25*, 41-846.

Joris, L., Dab, I., Quinton, P. M. Elemental composition of human airway surface fluid in healthy and diseased airways. *Am. Rev. Respir. Dis.* 1993, *148,* 1633–1637.

Joseph, A., Philip, R. Acute salinity stress alters the haemolymph metabolic profile of *Penaeus monodon* and reduces immunocompetence to white spot syndrome virus infection. *Aquaculture* 2007, *272*, 87-97.

Junger, W., Liu, F.C., Loomis, W.H., Hoyt, D.B. Hypertonic saline enhances cellular immune function. *Circ. Shock,* 1994, *42*, 190-196.

Junger, W., Rhind, S.G. , Rizoli, S.B., Cuschieri, J., Shiu, M.Y., Baker, A.J., Li, L., Shek, P.N., Hoyt, D.B., Bulger, E.M. Resuscitation of traumatic hemorrhagic shock patients with hypertonic saline - without dextran - inhibits neutrophil and endothelial cell activation. *Shock*, 2012, *38(4),* 341-350.

Kalujnaia , S., MC William, I., Zaguinaiko, V., Feilen, A., Nicholson, J., Hazon, N., Cutler, C., Cramb, G. Trascriptomic approach to the study of osmoregulation in the European eel *Anguilla anguillaI. Physiol Genomics,* 2007, *31,* 385-401.

Kannel, W.B. Some lessons in cardiovascular epidemiology from Framingham. *Am. J. Cardiol.*, 1976, *37*, 269–82.

Kolsen-Petersen, J.A. Immune effect of hypertonic saline: fact or fiction? *Acta Anaesthesiol. Scand.,* 2004, *48,* 667-678.

Kolsen-Petersen, J.A., Nielsen, J.O., Tonnesen, E.M. Effect of hypertonic saline infusion on postoperative cellular immune functions. *Anesthesiology*, 2004, *100*, 1108-1118.

Kültz, D., Avila K. Mitogen-activated protein kinases are in vivo transducers of osmosensory signals in fish gill cells. *Comp. Biochem. Physiol. B,* 2011, *129*, 821-829.

Kültz, D., Li, J., Gardell, A., Sacchi, R. Quantitative molecular phenotyping of gill remodelling of gill in a cichlid fish responding to salinity stress. *Mol. Cell. Proteomics,* 2013, *12*, 3962–3975.

Lafyatis, R., Farina, A. New insights into the mechanisms of innate receptor signaling in fibrosis. *Open Rheumatol*., 2006, *6*, 72-79.

Lai, K.P., Li, J.W. , Gu, J. , Chan, T .F. , Tse, W.K. , Wong, C.K. Transcriptomic analysis reveals specific osmoregulatory adaptive responses in gill mitochondria-rich cells and pavement cells of the Japanese eel. *BMC Genomics, 2015*, *16,* 1072.

Laing, K.J., Purcell, M.K., Winton, J.R., Hansen, J.D. A genomic view of the NOD like receptor family in teleost fish: identification of a novel NLR subfamily in zebrafish. *BMC Evol. Biol*., *2008,* 8, 1471-2148.

Laing, K.J., Hansen, J.D. Fish T cells: Recent advances through genomics. *Dev. Comp. Immunol.,* 2011, *35(12),* 1282-1295.

Latta, L.C., Weider, L.J., Colbourne, J.K., Pfrender, M.E. The evolution of salinity tolerance in Daphnia: a functional genomics approach. *Ecol. Lett*., 2012, *15,* 794-802.

Le Moullac, G., Haffner, P. Environmental factors affecting immune responses in crustacea. *Aquaculture*, 2000, *191*, 121–131.

Lenz, M., da Gama, B.., Gerner, N.V. , Gobin, J. , Gröner, F., Harry, A. , Jenkins, S. , Kraufvelin, P., Mummelthei, C. , Sareyka, J., Xavier, E., Wahl, M. Non-native marine invertebrates are more tolerant towards environmental stress than taxonomically related native species: Results from a globally replicated study. *Environ. Res.*, 2011, *111*, 943-952.

Li, D.Q., Luo, L., Chen, Z., Kim, H.S., Song, X.J., Pflugfelder, S.C. JNK and ERK MAP kinases mediate induction of IL-1β, TNF-α and IL-8 following hyperosmolar stress in human limbal epithelial cells. *Exp Eye Res*. 2006, *82,* 588–596.

Lin, Y. C., Chen, J.C., Li, C., Morni, Z.W., Suhaili, A.S, Kuo, Y.H., Chang, Y.H., Chen, L.L., Tsui, W.C., Chen, Y., Huang, C.L... Modulation of the innate immune system in white shrimp *Litopenaeus vannamei* following long-term low salinity exposure. *Fish Shellfish Immunol.,* 2012, *33,* 324-31.

Loomis, W.H., Namiki, S., Hoydt, D.B., Junger, W. Hypertonicity rescues T cells from suppression by trauma-induced anti-inflammatory mediators. *Am. J. Physiol. Cell. Physiol*., 2001, *281*, C840-848.

Loomis, W.H., Namiki, S., Ostrom, R., Insel, P.A., Junger, W. Hypertonic stress increase T cell interleukin-2 expression through a mechanism that involves ATP release, P2 receptor and p38 MAPK activation. *J. Biol. Chem.*, 2003, *278(7),* 4590-4596.

Lopez-Rodriguez, C., Aramburu, J., Jin, L., Rakeman, A., Michino, M., Rao, A. Bridging the NFAT and NF-kB Families: NFAT5 dimerization regulates cytokine expression gene transcription in response to osmotic stress. *Immunity*, 2010, *15,* 47-58.

Lu, Y. Q., Cai, X. J., Gu, L. H., Mu, H. Z., Huang, W. D. Hypertonic saline resuscitation maintains a more balanced profile of T-lymphocytes subpopulations in a rat model of hemorrhagic shock. *J. Zhejiang Univ. Sci. B*, 2007, *8(1*), 70-75.

Lütfioğlu, M., Sakallıoğlu, E. E., Sakallıoğlu, U., Çiftçi, G., Diraman, E. Osmotic pressure of gingiva in periodontitis: correlation with gingival proinflammatory cytokine production and alveolar bone destruction. *J. Dent. Sci.*, 2011, *6*(1), 19-24.

Ma, Z., Zheng, P., Guo, H., Jiang, S., Qin, J.G., Zhang, D., Liu, X. Salinity regulates oxidant enzyme and Na^+K^+-ATPase activities of juveniles golden pomprano *Trachinotus ovatusi* (Linnaeus 1758). *Aquaculture Res*., 2014, *47*(5), 1481-1487.

Machnik, A., Neuhofer, W., Jantsch, J., Dahlmann, A., Tammela, T., Machura, K., Park, J., Beck, F.X., Muller, D.N., Derer, W., Goss, J., Ziomber, A., Dietsch, P., Wagner, H., van Rooijen, N., Kurtz, A., Hilgers, K.F., Alitalo, K., Eckardt, K.U., Luft, F., Kerjaschki, D., Titze, J. Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanisms. *Nat. Med.*, 2009, *15*, 545-552.

Magor, B.G. Antibody affinity maturation in fish – our current understanding. *Biology* 2015, 4, 512-524.

Marc, AM., Quentel, C., Severe, A., Le Bail, P.Y., Boeuf, G. Changes in some endocrinological and non-specific immunological parameters during seawater exposure in the brown trout. *J. Fish Biol.,* 1995, *46*, 1065-1081.

Maryoung, L.A., Lavado, R., Bammler, T., Gallagher, E., Stapleton, P., Beyer, R., Farin, F., Hardiman, G., Schlenk, D. Differential Gene Expression in Liver, Gill, and Olfactory Rosettes of Coho Salmon (*Oncorhynchus kisutch*) After Acclimation to Salinity. *Mar. Biotechnol.* 2015, *17(6),* 703-717.

Matozzo, V. , Chinellato, A. , Munari, M. , Finos, L. , Bressan, M. , Marin, M. G. First evidence of immunomodulation in bivalves under seawater acidification and increased temperature. *PLoS One*, 2012, *7*(3), e33820.

Medzhitov, R., Janeway, C.A. Innate immune recognition: mechanisms and pathways. *Immunol. Rev*., 2000, *173,* 89-97.

Medzhitof, R. Recognition of microorganisms and activation of the immune response. *Nature*, 2007, *449*, 819-826.

Morrison, R., Nowak, B. The antibody response of teleost fish. *Sem. Av. Ex. P. Med.,* 2002, *11*, 46-54.

Murao, Y., Hoyt, D. B., Loomis, W., Namiki, S., Patel, N., Wolf, P., Junger, W. G. (2000). Does the timing of hypertonic saline resuscitation affect its potential to prevent lung damage ? *Shock, 2000, 14*(1) 18-23.

Nemeth, Z.H., Deitch, E.A., Szabo, C., Hasko, G. Hyperosmotic stress induces nuclear factor-κB activation and interleukin-8 production in human intestinal epithelial cells. *Am. J. Pathol.,* 2002, *161*, 987–96.

Neuhofer, W. Role of NFAT5 in inflammatory disorders associated with osmotic stress. Curr. Genomics, 2010, *11,* 584-590.

Ogryzko, N.V., Renshaw, S.A., Wilson, H.L. The IL1 family in fish: swimming through the muddy waters of inflammasome evolution. *Dev Comp Immunol*. 2014, *46(1),* 53-62.

Oreopoulos, G. D., Bradwell, S., Lu, Z., Fan, J., Khadaroo, R., Marshall, J. C., Rotstein, O. D. Synergistic induction of IL-10 by hypertonic saline solution and lipopolysaccharides in murine peritoneal macrophages. *Surgery*, 2001, *130*(2), 157-165.

Ori, D., Kato, H., Sanjo, H., Tartey, S., Mino, T., Akira, S., Takeuchi, O. Essential roles of K63-linked polyubiquitin-binding proteins TAB2 and TAB3 in B cell activation via MAPKs. *J. Immunol*. 2013, *190(8*), 4037-45.

Oseko, N. Occurrence and prevention of white spot syndrome (WSSV) in Malaysia. *Proceedings from the 32nd edition, National Research Institute of Aquaculture*, 2006, 422-31.

Pancer, Z., Amemiya, C., Ehrhardt, G., Ceitlin, J., Gartland, G., Cooper, M. Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. Nature, 2004, *430*, 174-180.

Pancer, Z., Cooper, M.D. The evolution of adaptive immunity. *Annu. Rev. Immunol.,* 2006, *24*, 497-518.

Pan, Z., Wang, Z., Yang, H., Zhang, F., Reinach, P.S. TRPV1 activation is required for hypertonicity stimulated inflammatory cytokine release in human corneal epithelial cells. *Invest. Ophthalmol. Vis.,* 2011, *52*, 485–493.

Perrigault, M., Buggé, D., Allam, B. Effect of environmental factors on survival and growth of Quahog Parasite Unknown (QPX) *in vitro. J. Invertebr. Pathol*. 2010, *104*, 83–89

Perrigault, M., Dahl, S.F. , Espinosa, E.P. , Allam, B. Effects of salinity on hard clam (*Mercenaria mercenaria*) defence parameters and QPX disease dynamics. *J. Invertebr. Pathol.,* 2012, *110,* 73–82.

Petersen, S.V., Thiel, S., Jensen, L., Steffensen, R., Jensenius, J.C. An assay for the mannan-binding lectin pathway of complement activation. *J. Immunol. Meth*., 2001, *257*, 107-116.

Plouffe D. A., Hanington P. C., Walsh J. G., Wilson E. C. & Belosevic M. (2005) Comparison of select innate immune mecha- nisms of fish and mammals. *Xenotransplantation* 12 : 266–77.

Prayitno, S.B., Latchford, J.W. Experimental infections of crustaceans with luminous bacteria related to *Photobacterium* and *Vibrio*. Effect of salinity and pH on infectivity. *Aquaculture*, 1995, *132,*105–112

Rauta, P. R., Nayak, B. , Das, S. Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. *Immunol. Lett.*, 2012, *148*(1), 23-33.

Reid, H.I., Soudant, P.I., Lambert, C., Paillard, C., Birkbeck, T.H. Salinity effects on immune parameters of *Ruditapes philippinarum* challenged with *Vibrio tapetis*. *Dis. Aquat. Organ*., 2003, *56*, 249-258.

Rizoli, S. B., Rhind, S. G., Shek, P. N., Inaba, K., Filips, D., Tien, H., Brennaman, F., Rotstein, O. The immunomodulatory effects of hypertonic saline resuscitation in patients sustaining traumatic hemorrhagic shock: a randomized, controlled, double-blinded trial. *Ann. Surg*., 2006*, 243*(1), 47-57.

Schatz, D. G., Oettinger, M. A., Schlissel, M. S. V(D)J recombination: molecular biology and regulation. *Annu. Rev. Immunol*., 1992, *10*, 359–383.

Schilli, R., Breuer, R.I., Klein, F., Dunn, K., Gnaedinger, A., Bernstein, J., Paige, M., Kaufman, M. Comparison of the composition of faecal fluid in Crohn's disease and ulcerative colitis. *Gut*, 1982, *23,* 326–32.

Schwartz, L., Abolhassani, M., Pooya, M., Steyaert, J.M., Wertz, X., Israël, M., Guais, A., Chaumet-Riffaud, P. Hyperosmotic stress contributes to mouse colonic inflammation

through the methylation of protein phosphatase 2A. *Am. J. Physiol. Gastrointest. Liver Physiol.*, 2008, *295(5),* G934-41.

Schwartz, L., Guais, A., Pooya, M., Abolhassani, M. Is inflammation a consequence of extracellular hyperosmolarity? *J. Inflamm*., 2009, *6*(21).

Shapiro, L., Dinarello, C.A. Hyperosmotic stress as a stimulant for proinflammatory cytokine production. *Exp. Cell. Res*., 1997, *231*, 354-362.

Smith, M. J., Schreiber, E. S. G., Scroggie, M. P., Kohout, M., Ough, K., Potts, J., Lennie, R., Turnbull, D., Jin, C., Clancy, T. I. M. Associations between anuran tadpoles and salinity in a landscape mosaic of wetlands impacted by secondary salinisation. *Freshwater Biology*, *2007, 52*(1), 75-84.

Son, V.N., Phuong, N., Hai, T.N., Yakupitiyage, A. Production and economic efficiencies of intensive tiger prawns (Penaeus monodon) culture during different cropping seasons in the Mekong Delta. *Aquacult. Int*., 2010, *19*, 555-566.

Stookey, J.D., Pieper, C.F., Cohen, H.J. Hypertonic hyperglycemia progresses to diabetes faster than normotonic hyperglycemia. *Eur. J. Epidemiol*., 2004*, 19*, 935–944.

Strugnell, R.A., Wijburg, O.L. The role of secretory antibodies in infection immunity. *Nat. Rev. Microbiol*., 2010, *8*, 656-667.

Sunyer, J.O., Lambris J.D. Diversity of the third form of complement, C3, in fish: functional characterization of five forms of C3 in the diploid fish *Sparus aurata*. *Biochem. J.,* 1997, *326*, 877-881.

Sunyer, J.O., Tort, L. Natural haemolytic and bactericidal activities of sea bream Sparus aurata are effected by the alternative complement pathway. *Vet. Immunol. Immunopathol.,* 1995, *45*, 333-345.

Sunyer, J.O. Evolutionary and Functional Relationships of B Cells from Fish and Mammals: Insights into their Novel Roles in Phagocytosis and Presentation of Particulate Antigen. Infect. Disord. *Drug Targets*, 2012, *12(3)*, 200-212.

Surh, C. D., Sprent J. Homeostasis of naive and memory T cells. *Immunity*, 2008, *29,* 848–862

Tort, L., Balasch, J.C., Mc Kenzie, S. Fish immune system. A crossroads between innate and adaptive response. *Immunologia*, 2003, *22(3)*, 277-286.

Uribe, C., Folch, H., Enriquez, R., Moran, G. Innate and adaptive immunity in teleost fish: a review. *Vet. Med*., 2011, 56(10), 486-503.

Vaseeharan, B., Ramasamy, P., Wesley, S. G., Chen, J. C. Influence of acute salinity changes on biochemical, haematological and immune characteristics of *Fenneropenaeus indicus* during white spot syndrome virus challenge. *Microbiol. Immunol*., 2013,*57*, 463-469.

Vatanavicharn, T., Supungul, P., Puanglarp, N. Yingvilasprasert, W., Tassanakajon, A. Genomic structure, expression pattern and functional characterization of crustinPm5, a unique isoform of crustin from Panaeus monodon. *Comp. Biochem. Phys. B,* 2009, *153*, 244-252.

Vargas-Albores, E., Baltazar, P.H., Clark, G., Barajas, F. Influence of temperature and salinity on the yellowleg shrimp, *Penaeus californiensis* Holmes prophenoloxidase system. *Aquaculture Res*., 1998, *28*, 549–553

Wang, L. U., Chen, J.C.. The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* at different salinity levels. *Fish Shellfish Immunol.,* 2005, *18,* 269–278.

Wang, L.U., Chen J.C. Effect of salinity on the immune response of tiger shrimp *Penaeus monodon* and its susceptibility to *Photobacterium damselae* subsp. *Damselae. Fish Shellfish Immunol.,* 2006, *20, 671-681.*

Wang, F., Yang, H., Gao, F., Liu, G.. Effects of acute temperature and salinity stress on the immune response in sea cucumber *Apostichopus japonicus*. *Comp. Biochem. Phys. A,* 2008, *151*, 491-498.

Watson, F.L., Püttmann-Holgado, R., Thomas, F., Lamar, D.L., Hughes, M., Kondo, M., Rebel, V.I., Schmucker, D. Extensive diversity of Ig superfamily proteins in the immune systems of insects. *Science*, 2005, *309*, 1874-1878.

Whittington, R. J., Munday, B. L., Akhlaghi, M., Reddacliff, G. L., Carson, J. Humoral and peritoneal cell responses of rainbow trout (*Oncorhynchus mykiss*) to ovalbumin, *Vibrio anguillarum* and Freund's complete adjuvant following intraperitoneal and bath immunisation. *Fish Shellfish Immunol*., 1994, *4*, 475-488.

Woehrle, T., Yip, L., Manohar, M., Sumi, Y., Yao, Y., Chen, Y., Junger, W. G. Hypertonic stress regulates T cell function via pannexin-1 hemichannels and P2X receptors. *J. Leukoc. Biol.*, 2010, *88*(6), 1181-1189.

Yada, T., Azuma, T., Takaji, Y. Stimulation of non-specific immune functions in seawater-acclimated rainbow trout *Oncorhynchus mykiss*, with reference to the role of growth hormone. *Comp. Biochem. Phys. B*, 2001, *129*, 695-701.

Yancey, P.H. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J. Exp. Biol.,* 2005, *208*, 2819-2830.

Ye, J., Bromage, E.S., Kaattari, S.L. The strength of B cell interaction with antigen determines the degree of IgM polymerization. *J. Immunol*. 2010, *184*, 844–850.

Yu, Z.,, Li, C., Guan, Y. Effect of salinity on the immune responses and outbreak of white spot syndrome in the shrimp *Marsipenaeus japonicas*. *Ophelia,* 2003, *57*, 99-106.

Zarejabad, A. M., Jalali, M. A., Sudagar, M., Pouralimotlagh, S. Hematology of great sturgeon (*Huso huso*) juvenile exposed to brackish water environment. *Fish Physiol. Biochem*., 2003, *36(3),* 655-659.

Zhang, Y.A. , Hikima, J. , Li, J. , LaPatra, S.E., Luo, Y.P., Sunyer, J.O. Conservation of structural and functional features in a primordial CD80/86 molecule from rainbow trout (*Oncorhynchus mykiss*), a primitive teleost fish. *J. Immunol.,* 2009, *183(1)*, 83–96.

Zhu, L.Y., Lin, A.F., Shao, T., Nie, L., Dong, W.R., Xiang, L.X., Shao, J.Z. B cells in teleost fish act as pivotal initiating APCs in priming adaptive immunity: an evolutionary perspective on the origin of the B-1 cell subset and B7 molecules. *J. Immunol*. 2014, *192(6),* 2699-714.

Zimmerman, L. M., Vogel, L. A., Bowden, R. M. Understanding the vertebrate immune system: insight from the reptilian perspective. *J. Exp. Biol*., 2009,*213,* 661-671.

Chapter 2

Osmoregulatory and immunological status of the pond-raised striped catfish (*Pangasianodon hypophthalmus* S.) as affected by seasonal runoff and salinity changes in the Mekong Delta, Vietnam.

Schmitz, M.; Baekelandt, S.; Lam Khoa, T.; Mandiki, S.N.M.; Douxfils, J.; Thinh, N.; Huong, D.T.T.; Kestemont, K. Osmoregulatory and immunological status of the pond-raised striped catfish (*Pangasianodon hypophthalmus, S.*) as affected by seasonal runoff and salinity changes in the Mekong Delta, Vietnam. *Fish Physiol Biochem*., 2016, *DOI 10.1007/s10695-016-0266-7.*

Abstract

In Vietnam, the production of striped catfish *Pangasianodon hypophthalmus* S. reached more than 1.2 millions of tons in 2014 and is mainly gathered in the Mekong Delta (South Vietnam). A survey was carried out during the dry season 2013 (March-April) and rainy season 2014 (July-August) in 12 fish farms of the lower (Tra Vinh Province) and higher (Can Tho Province) Mekong River Delta. This study allowed drawing up current key osmoregulatory and innate immune parameters of striped catfish in aquaculture depending on the geographical location, particularly the proximity to the River Mekong Estuary and the seasonal surface runoff. In the dry season, plasma osmolality was positively correlated with salinity. Gill Na^+K^+ ATPase dropped in the rainy season while kidney Na^+K^+ ATPase remained stable. Abundance of immune blood cells, especially thrombocytes and monocytes, tended to increase in farms located in tidal area. Production of reactive oxygen species in the spleen, kidney lysozyme activity and plasma complement activity did not vary whatever the season or the proximity to the estuary. Plasma lysozyme activity was 50-fold higher than in kidney and increased in the rainy season as well as in tidal sites. Kidney complement activity decreased in the dry season, especially in tidal sites. In conclusion, regarding key osmoregulatory and immune factors, striped catfish farms located in the Mekong Delta may be affected by seasonal and regional salinity and runoff fluctuations.

1. Introduction

In Vietnam, striped catfish production reached 1.2 millions of tons in 2014 with an estimated value of 1.77 billion US$ in the international food market, representing almost 80 % of the total world production of this species (FAO, 2014; VASEP, 2015). The Mekong Delta, which actually includes 77 % of fisheries Vietnamese production, is a region rich in aquatic resources with high potential for agricultural development (VASEP, 2015). In the Mekong Delta, catfish farms typically consist on average 4-m deep ponds, ranging in size from 0.035 – 0.1 ha for small scale farms to more than 0.3 ha for large scale farms (Bosma et al.,2009; De Silva and Phuong, 2011). Given to high organic load, high stocking density and absence of aeration, oxygen is restricted to the top 1 m of the water column during the day, while ponds can be completely anoxic during the night (Lefevre et al.,2011a). However, high tolerance to nitrite

(LC50 96 h: 1.95 mM) (Lefevre et al.,2011b) and salinity (up to 15 ppt) (Thanh et al.,2014), fast-growth and high capacity for aerial and aquatic oxygen uptake due to high developed gills and a modified swim bladder (< 2KPa) (Lefevre et al.,2011c) make easy the culture of the striped catfish.

The Mekong Basin is characterized by a tropical to subtropical monsoon climate, with a dry season from November to April and a rainy season from May to October. During the rainy season, water flow into the Mekong Delta may reach more than 20 000 $m^3 s^{-1}$ and flood 35 – 50 % of the delta each year (MRC, 2010). Mean suspended solid concentrations reach 68.3 mg L^{-1} during the rainy season compared with 31.8 mg L^{-1} during the dry season (Isobé et al.,2003). With the onset of the rainy season, a higher occurrence of infectious diseases and parasites is usually recorded in catfish and prawns farms (Phan et al., 2009; Son et al., 2010). During the dry season, decrease in water flow to less than 5 000 $m^3 s^{-1}$ induces saltwater intrusion from the China Sea and the Gulf of Thailand into many coastal provinces of the Mekong Delta (MRC, 2010). In February 2016, salinity intrusion to inland reach 90 km to mouth and salinity up to 12 ppt was measured in several aquaculture provinces (SIWRR, 2016). In the tidal range, the mixing of seawater and freshwater induces modification in water circulation and help to retain nutrients (MRC, 2010). Phan et al.,(2009) have already reported lower yield in catfish farms located in the tidal range although Phuc et al.,(2014) revealed no significant effect of salinity level from freshwater to 10 ppt on fish weigh gain or specific growth rate. Over the next century, saltwater intrusions will be exacerbated in the Mekong delta, by the sea level rise, which will be comprised between 0.26 and 0.98 m (IPCC, 2013) as well as by the decreased water flow, due to dam construction (especially in China) and intensive pumping for irrigation in Thailand.

Environmental factors are tightly associated to immune changes and occurrence of diseases. Therefore, it is of critical importance to study the range of tolerance to environmental changes for aquaculture species. The innate response is a fundamental primary defence in fish (Magnadottir, 2006; Uribe, 2011). Humoral innate parameters, in particular phagocytic cells, lysozyme and complement activities, have been extensively used as indicators of the effects of external factors in the immune system or disease resistance (Magnadottir, 2006). Osmotic stress and suspended solids have been associated to important changes in immune parameters. In Nile Tilapia *Oreochromis niloticus* L., gradual increase in suspended sediments from 0 to 2000 mg L^{-1} induced a gradual elevation of plasma lysozyme activity (Dominguez et al.,2005). In Mozambic tilapia *Oreochromis mossambicus* P., transfer from freshwater to brackish water increased the lysozyme activity 1 and 24 h after transfer as well as alternative complement activity and reactive oxygen species production in kidney and spleen 8 h after transfer (Jiang et al.,2008). In rainbow trout *Oncorhynchus mykiss* L. and Nile tilapia, hyperosmotic stress induced elevated lysozyme activity in plasma (Yada et al.,2000; Dominguez et al.,2005). In grouper fry of *Epinephelus sp.,* hypo- or hyperosmotic shocks increased the susceptibility to infectious pancreatic necrosis virus (IPNV) (Chou et al.,1999).

Although this species is one of the most worldwide cultured species, to our knowledge there is no reference data available neither on the osmoregulatory status nor on the immune system of striped catfish in aquaculture. Therefore, this study aims to describe the immune status of striped catfish in actual aquaculture ponds in South Vietnam and associate seasonal/spatial immune changes to environmental variables, especially salinity intrusion in the river mouth. In total, 12 catfish farms sites were sampled upstream and downstream the tidal range in the Mekong Delta in the dry season (2013) and the rainy season (2014). The selected parameters focus on the osmoregulatory capacity of striped catfish and key innate immune defences including immune blood cells, production of reactive oxygen species (ROS) lysozyme and complement activities in plasma and tissues. First, we suspected that saline intrusions provide an osmotic stressor that changes the immunological status of the species in tidal sites, especially

during the dry season. Then, we hypothesized that runoff during the rainy season increases the sediment load and hence impacts the immunological status of the species in its aquaculture.

2. Materials and methods

2.1 Field samplings

The Mekong River, with a mean discharge of 14 500 m^3s^{-1} is the 12th highest river in the world (MRC, 2010). In Southeast Asia, it flows from the Tibetan Plateau through six countries and splits into 7 major branches in Vietnam up to the South China Sea. In South Vietnam, two sampling campaigns were carried out in March – April 2013 (dry season) and July – August 2014 (rainy season) in catfish growing farms sites through the Mekong Delta. In each season, 3 tidal sites (Tra Vinh Province, 40 km to mouth) and 3 upstream sites (125 km to mouth) were selected. During the dry season, three farm sites were randomly sampled in Tra Vinh Province (GPS data [9.964; 106.389]; [9.964; 106.388]; [9.961; 106.384]) and three farms sites in Can Tho Province ([10.291; 105.502]; [10.292; 105.501]; [10.291; 105.516]). During the rainy season, three other farm sites were randomly selected in Tra Vinh Province ([9.929; 106.271]; [9.752; 106.145]; [9.762; 106.204]) and Can Tho Province ([10.218; 105.592]; [10.193; 105.598]; [10.194; 105.568]).

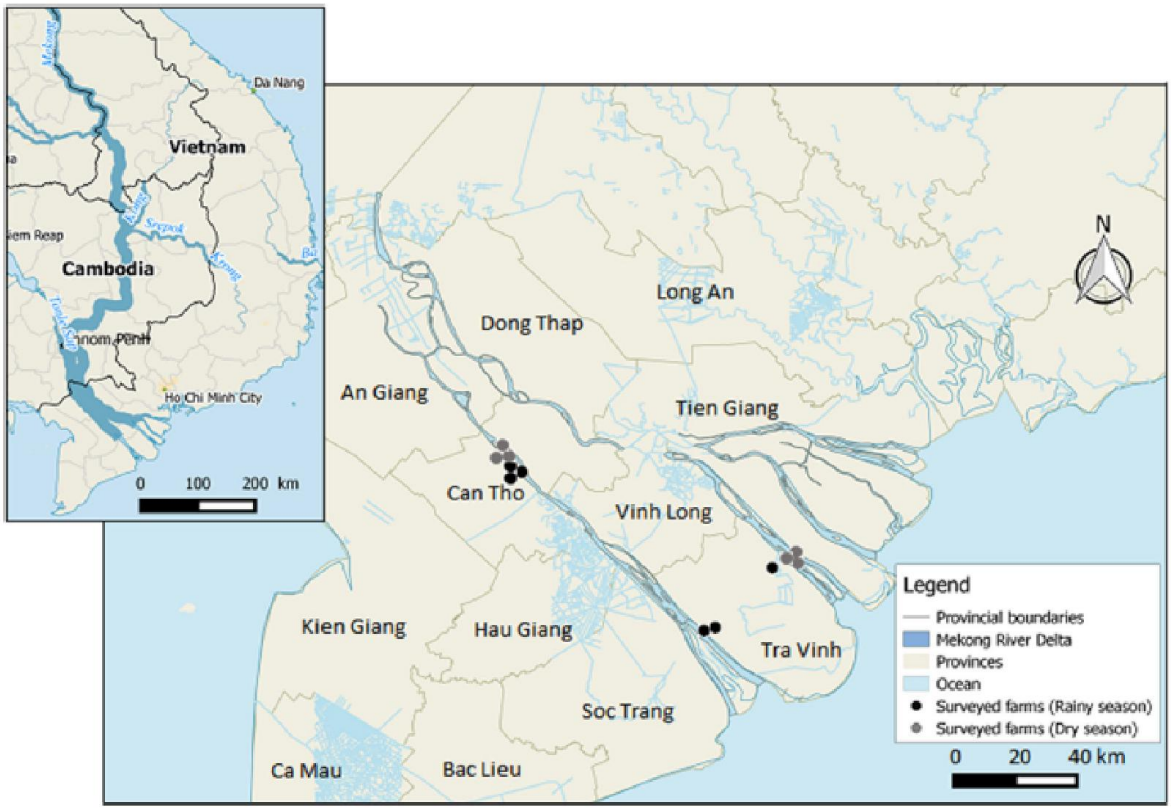


Figure 1: Mekong River Delta. Mekong River Delta and farm sites of surveyed striped catfish farms during the rainy (black symbol) and the dry (grey symbol) seasons.

Figure 1 shows the farm sites of surveyed striped catfish farms. Sampling times and farm sites were selected by local authorities, which did not allow us to sample the same farms in both seasons. The selected farms were characterized by area ranging between 1 000 and 16 000 m^2 with an average depth of 4 m and stocking density in ponds from 10 to 60 fish m^{-3} (3 to 8 kg

fish m^{-3}). Physicochemical data, including salinity, temperature, pH and dissolved oxygen, were measured during samplings at the water surface of the pond with a multiparameter probe (WTW, Multi 350i) (**Table 1**).

Table 1: Physicochemical variables in surveyed striped catfish farms. Physicochemical variables (temperature, salinity, pH and dissolved oxygen (DO)) measured in growing catfish farms sampled during March-April 2013 (dry season) and July-August 2014 (rainy season) in Tra Vinh and Can Tho Provinces. Results represented the mean±SEM of 3 independent farms. Values not sharing a common letter are significantly different ($p<0.05$).

	Dry season		**Rainy season**	
	Low Delta	High Delta	Low Delta	High Delta
Temperature (°C)*	31.6±0.2	32.5±0.5	31.2±0.3	29.4±0.4
Salinity (ppt)	$7±1.2^{a}$	$0±0^{b}$	$0±0^{b}$	$0±0^{b}$
pH**	7.3±0.4	6.6±0.2	8.1±0.3	8.1±0.5
DO (mg L^{-1})	4.5±1.1	2.3±0.3	6.5±2.7	4.1±1.0

*Temperature: ANOVA 2 reveals a statistical effect of season and farm sites but temperature may depend on the time of sampling.
**pH: ANOVA 2 reveals a statistical effect of season.

On each farm, 20 juveniles (100-200 g) were collected by the farmers with nets and anaesthetized in clove oil at 100 mg L^{-1} from a stock solution of 1g L^{-1} diluted 1:10 in 95% ethanol. Blood was collected by caudal vein puncture using a 1 ml heparinized syringe and stocked on ice during transport, until red blood cell counting few hours after fish bleeding. Fish were killed before organ removal by cervical dislocation. Gills (left arches 1 and 2) and whole kidney were taken out and immediately frozen in liquid nitrogen. The spleen was stocked on ice in L-15 media during transport and served for respiratory burst assay carried out the same day. Blood was centrifuged at 6 000 rpm during 10 min to collect plasma. Plasma and organs were frozen in dry ice to Belgium for analysis.
Monthly mean salinity data were collected by local authorities in Chau Thanh District, Tra Vinh City and Duc My District in the Co Chien River and in Cau Ké District, in the southern Bassac River between January 2011 and December 2013 (**Figure 2**). Maximal salinity values were measured in March 2013 at 11.6 ppt in Chau Thanh District (20 km to mouth), 2.4 ppt in Tra Vinh City (30 km to mouth) and Duc My District (40 km to mouth), and 0.2 ppt in Cau Ké District (40 km to mouth). In addition, mean water levels (gauge height) in the Mekong Delta, measured in Chau Doc district, are shown in **Figure 2** (GRDC, 2015).

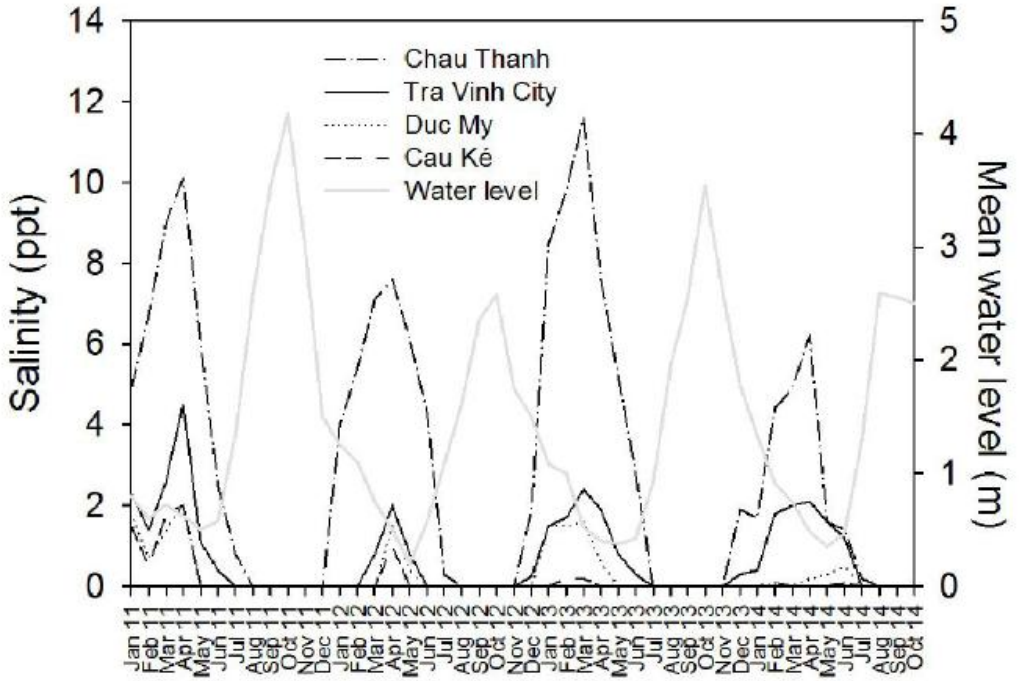


Figure 2: Seasonal salinity and water level changes in the Mekong Delta. Salinity (ppt) fluctuations per month between January 2011 and December 2013 in Chau Thanh district (20 km to mouth), Tra Vinh City (30 km to mouth), Duc My district (40 km to mouth) and Cau Ké district (40 km to mouth) in the Mekong Delta, Vietnam. Water level (gauge height in meters) per month between January 2011 and December 2013 measured in Chau Doc district.

2.2 Osmoregulatory parameters

Plasma osmolality

Plasma osmolality (15 µl) was measured by vapour pressure osmometer (Fisk One – Ten osmometer, model 210) in disposal tubes in duplicates.

Gill and kidney Na^+K^+ ATPase

Gill and kidney lysates were obtained by homogenizing for 2X30 s in ice cold SEI buffer (saccharose 0.25 M, imidazole 50 mM, EDTA 1 mM) pH 6.2 (kidney), pH 7.4 (gill) 1:5 containing protease inhibitor cocktail (Sigma) using Speed Mill Vac Bound Homogenizer. Main debris were removed by 2 successive centrifugations at 10 000 g during 5 min at 4°C. One aliquot (50 µl) was used to measure the Na^+K^+ ATPase activity according to the method of Mc Cormick (1993). One unit of Na^+K^+ ATPase activity represents the consumption of 1 µmole NADH min^{-1} ml^{-1}.

2.3 Blood cell populations

Red blood cells were counted on Neubauer hemocytometer by light microscopy. Briefly, 10 µL of each blood sample were diluted into 1990 µL Hanks Balanced Salt Solution (Sigma) and mixed gently for at least 3 min. Red blood cells were counted in 5 of the 25 small areas 0.1 mm^2. Samples were counted in duplicates. For leukocyte count, a small drop of whole blood was smeared on a microscope slide by using a smearing glass immediately after blood samplings. The slide smear was dried during 5 min at room temperature, fixed 1 min in methanol and finally stained with May-Grunwald Giemsa stain. Leukocytes subpopulations were determined following Vazquez et al.,(2007) by counting 1000 cells per slide.

2.4 Immune parameters

Spleen respiratory burst

Spleen respiratory burst was adapted from Rook et al.,(1985) and Choi et al.,(2006). This technique allows a quantification of intracellular ROS production by phagocytic cells including granulocytes and macrophages, although in fish, macrophages are the most actively phagocytic cells (Rook et al.,1985; Secombes and Fletcher, 1992; Choi et al.,2006). Spleen tissues were gently mashed into 500 µl L-15 medium using the back of a syringe piston and 100 mm nylon mesh grid into a Petri dish containing 500 µl of L-15 medium. The cell suspension was centrifuged at 500 g during 5 min. The supernatant was discarded and 500 µl of fresh L-15 medium was added under the cell suspension. This washing operation was performed twice time more. Aliquots (100 µl) of the cell suspension were then added to 1.5 ml polypropylene tubes in duplicates. Finally, 100 µl of nitroblue tetrazolium (NBT, 1 mg ml^{-1} in PBS, pH 7.4). After 1 h incubation at room temperature, samples were centrifuged at 5000 g during 5 min at 4°C and the supernatant was removed. The final pellets were washed in PBS, then once in methanol in order to discard extracellular NBT and air-dried at RT. The blue formazan in each tube was dissolved in 240 µl of KOH 2 M and 280 µl of N-dimethylformamide. After centrifugation at 3500 rpm during 10 min, 35 µl of final dissolved NBT was transferred to a 96-wells and absorbance was measured at 550 nm. Negative control stand for direct fixation of samples in methanol without incubation. The standard curve was done by directly dissolving serial dilution of NBT in KOH 2M and N-dimethylformamide.

Lysozyme activity

The lysozyme activity protocol was adapted from Ellis et al.,(1990). In flat bottom microplates 96 wells, the lysozyme activity assay was initiated by mixing 10 µl of kidney homogenate or 10 µl of plasma with 250 µl of lyophilized *Micrococcus lysodeikticus* (Sigma) suspension 0.6 mg ml^{-1} in phosphate buffer, pH 6.2. Kidney lysates were obtained by homogenizing for 2X30 s kidney tissue 1:5 in ice cold SEI buffer containing protease inhibitor cocktail (Sigma) pH 6.2 using Speed Mill Vac Bound Homogenizer. Main debris were removed by 2 successive centrifugations at 10 000 g during 5 min at 4°C. The difference in absorbance at 450 nm was monitored between 0 min and 15 min (linearity range). One unit of lysozyme activity represents the amount of lysozyme that causes a 0.001 decrease in absorbance min^{-1}. Samples were assayed in duplicate.

Alternative complement pathway

The alternative complement pathway was assayed from Sunyer and Tort (1995). In round bottom microplates 96 wells, 10 µl of rabbit red blood cells suspension (3% in veronal buffer) were mixed with serial dilutions of plasma or kidney lysate (70 µl of total volume). After incubation for 2 h at 27°C, the samples were centrifuged at 2000 g for 5 min at 4°C. Haemolysis 100% was obtained by adding 60 µl of distillate water to 10 µl of RRBC. Negative was obtained by adding 60 µl of veronal buffer to 10µl of RRBC. Finally, 35 µl of supernatant was measured at 405 nm absorbance. Samples were measured in duplicates. The ACH 50 value (unit ml^{-1} plasma) is defined as the reciprocal of the plasma dilution which induces the haemolysis of 50% RRBC.

2.5 Statistical analysis

Equal variance was tested by Levene test and normality was checked by Shapiro-Wilk test. We first performed a three-way analysis of variance (3 factors: “season”, “proximity to mouth” and “farm”) with n=3 that revealed heterogeneity between farms. Therefore, farms (n=3) have been considered as the experimental unit and the changes in osmoregulatory and immune parameters were analysed by two-way ANOVA (2 factors: “season”, “proximity to mouth”). ANOVA 2

was then followed with pairwise multiple comparisons procedures by non-parametric Tukey test (α=5%) as criterions for normality and / or homogeneity of variances were not always checked despite adequate transformations. Figures and statistical analysis were performed in Sigmastat. Farm sites were considered as the experimental unit (n=3). Data are represented by the mean ± SEM, n=3.

3. Results.

3.1 Osmoregulatory parameters

Plasma osmolality varied from 266 and 270 mosm in catfish raised outside of the tidal range and significantly increased in catfish raised in tidal sites during the dry season to reach about 282 mosm ($p<0.05$) (**Figure 3 A**). **Figure 3 B** shows plasma osmolality of each independent farms sampled during the dry season compared to the relative pond salinity. Gill Na^+K^+ ATPase activity was twice lower during the rainy season ($p<0.001$) ranging between 0.17 and 0.26 U g^{-1} tissue comparing to the dry season, where values ranged between 0.44 and 0.91 U g^{-1} tissue (**Figure 4**). Na^+K^+ ATPase activity was similar between the stations during the rainy season while in the dry season, Na^+K^+ ATPase activity was lower in farms located downstream (0.54±0.09 U g^{-1} tissue) compared to farms located upstream (0.79±0.08 U g^{-1} tissue) ($p<0.05$). In kidney, Na^+K^+ ATPase activities were generally more elevated than in gills and varied between 0.63 and 1.30 U g^{-1} tissue (**Figure 4**).

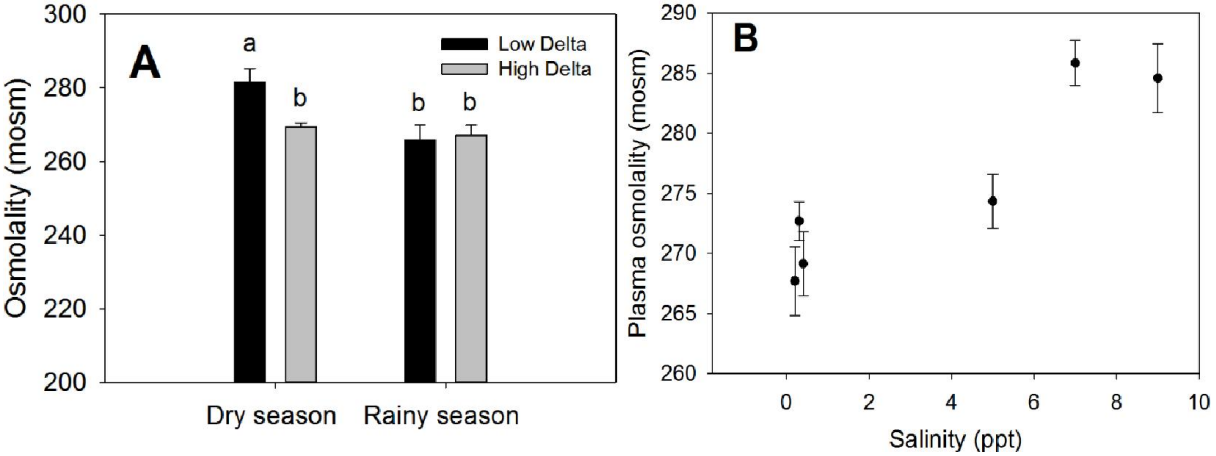


Figure 3: Plasma osmolality of striped catfish in aquaculture in the Mekong Delta. (A) Plasma osmolality of striped catfish *Pangasianodon hypophthamus* L. raised in farms located in the Low and High Delta, sampled during the dry and rainy seasons. Results represented the mean±SEM of 3 independent farms. Values not sharing a common letter are significantly different ($p<0.05$). (B) Plasma osmolality of 6 independent farm sampled during the dry season compared to the relative pond salinity. Results represented the mean per farm±SEM of 20 fish.

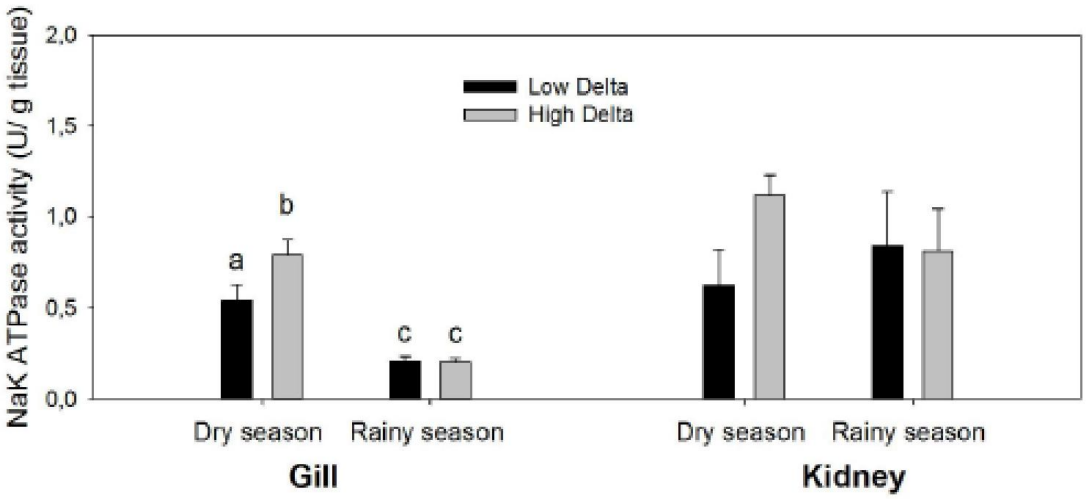


Figure 4: Osmoregulatory parameters of striped catfish in aquaculture in the Mekong Delta. Plasma osmolality (A) and Na^+K^+ ATPase activity in gill (B, left) and kidney (B, right) of striped catfish *Pangasianodon hypophthamus* L. raised in farms located in the Low and High Delta, sampled during the dry and rainy seasons. Results represented the mean±SEM of 3 independent farms. Values not sharing a common letter are significantly different ($p<0.05$).

3.2 Blood cell populations

Table 2 shows the variation of blood parameters according to the season and the proximity of the estuary. Red blood cell abundance significantly increased in tidal sites, ranging from 2.03 to 2.47 million cells μl^{-1} blood compared to 1.77 to 2.03 million cells μl^{-1} blood in non-tidal sites. Thrombocytes were the most abundant blood cells after erythrocytes and generally represented 80-85% of white blood cells+thrombocytes, followed by lymphocytes (10-15%), granulocytes (2.8-4.2%) and monocytes (1.8-4%). The abundance of thrombocytes was lower in the dry season (93 to 119 thousand cells μl^{-1} blood) than in the rainy season (128 to 134 thousand cells μl^{-1} blood) ($p<0.05$). The number of circulating monocytes significantly declined during the dry season ($p<0.001$). Indeed, during the dry season, abundance of monocytes averaged 2.31 thousand cells μl^{-1} blood in tidal sites and 2.11 thousand cells μl^{-1} blood in non-tidal sites while it reached 6.82 thousand cells μl^{-1} blood in tidal sites and 4.17 thousand cells μl^{-1} blood in non-tidal sites during the rainy season. Moreover, monocytes were slightly less abundant upstream in both season ($p<0.05$). By contrast, the number of blood lymphocytes and granulocytes did not vary significantly between geographical locations nor between seasons.

Table 2: Blood parameters of striped catfish in aquaculture in the Mekong Delta. Red blood cell counting (RBC), thrombocytes and absolute differential leukocyte count in Vietnamese catfish *Pangasianodon hypophthalmus* raised in farms located in the low and high Delta, sampled during the dry and the rainy season. Results represented the mean±SEM of 3 independent farms. Values not sharing a common letter are significantly different ($p<0.05$).

	Dry season		**Rainy season**	
	Low Delta	**High Delta**	**Low Delta**	**High Delta**
RBC ($x10^6$ μl^{-1})	2.21 ± 0.13^{a}	1.95 ± 0.04^{b}	2.25 ± 0.11^{a}	1.81 ± 0.03^{b}
Thrombocytes ($x10^3$ μl^{-1})	119.33 ± 5.24^{ab}	93.30 ± 5.15^{b}	134.19 ± 9.84^{a}	127.80 ± 11.04^{ab}
Lymphocytes ($x10^3$ μl^{-1})	17.58±8.79	17.38±8.69	18.60±2.40	19.53±1.31
Monocytes ($x10^3$ μl^{-1})	2.31 ± 0.64^{a}	2.11 ± 0.60^{a}	6.82 ± 0.72^{b}	4.17 ± 0.33^{c}
Granulocytes ($x10^3$ μl^{-1})	4.09±0.50	3.58±1.01	7.09±0.96	5.74±1.48

3.3 Immune parameters

Spleen respiratory burst (**Figure 5 A**) did not significantly vary whether the season or the proximity of the estuary and wavered between 6.55 and 12.0 mg formazan h^{-1} g^{-1} spleen. During the dry season, plasma lysozyme activity (**Figure 5 B**) significantly increased at 573±38 U ml^{-1} plasma in tidal sites compared with non-common sites (367±47 U ml^{-1}) ($p<0.01$). In the rainy season, lysozyme activity did not change significantly between tidal sites (536±50 U ml^{-1} plasma) and non-common sites (534±31U ml^{-1} plasma). Kidney lysozyme activity (**Figure 5 C**) corresponded to levels 50-fold lower than those assayed in plasma and did not show any significant change whether the season or the location. On the contrary, plasma and kidney ACH50 values were closed to each other (**Figure 5 D**). In plasma, mean ACH50 values ranged from 11.1±1.6 to 20.6±6.0. In kidney, ACH50 was 17.8±1.3 in tidal sites in the dry season and 14.7±0.6 upstream while during the rainy season ACH50 averaged 17.8 to 18.7. ACH50 values in kidney were significantly more elevated during the rainy season ($p<0.01$).

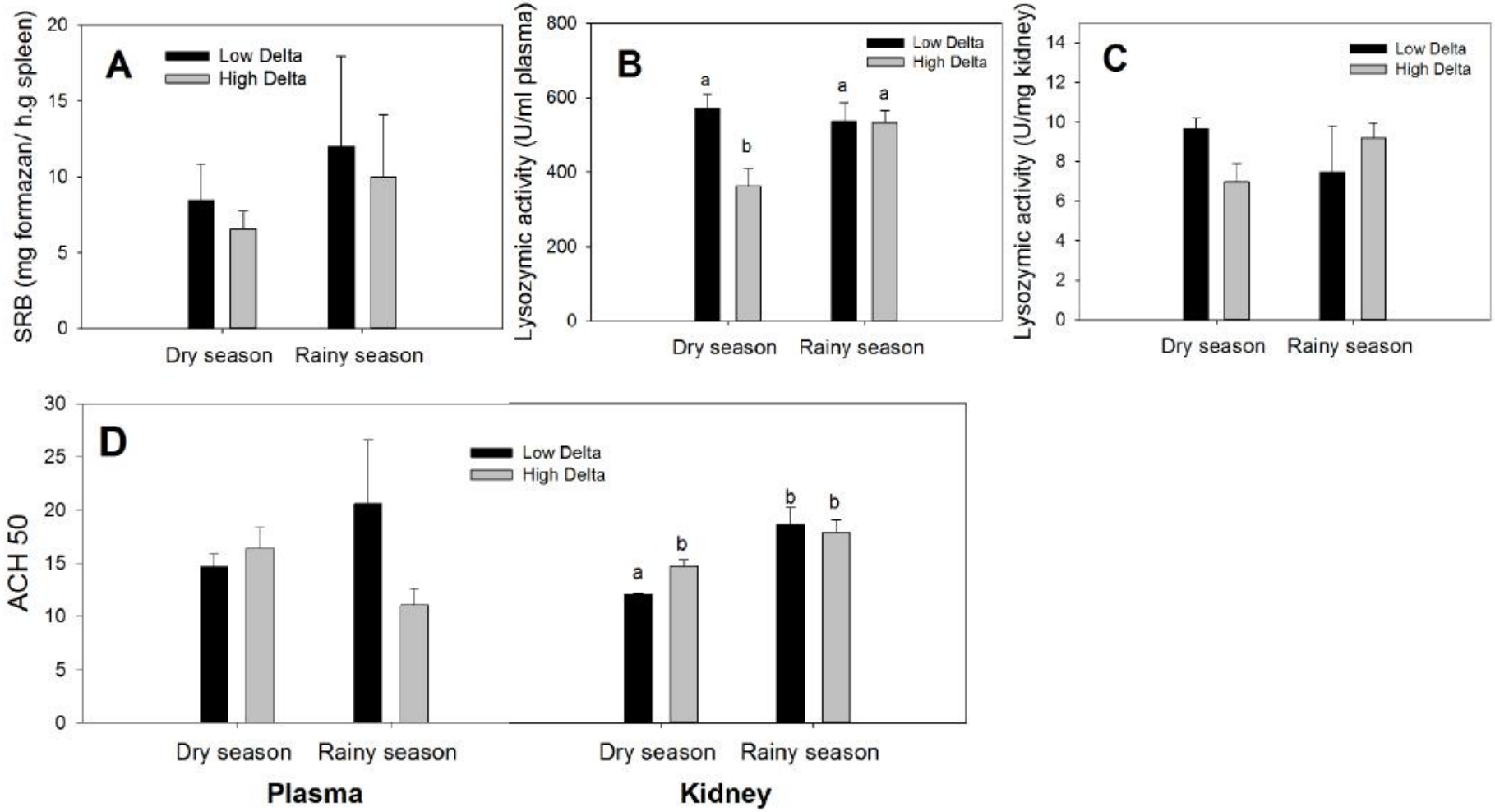


Figure 5: Immune parameters of striped catfish in aquaculture in the Mekong Delta. Spleen respiratory burst (A), lysozyme activity in the plasma (B), lysozyme activity in the anterior and posterior kidney (C) and alternative complement pathway activity (ACH 50) in plasma (D, left) and kidney (D, right) of striped catfish *Pangasianodon hypophthalmus* L. raised in farms located in the Low and High Mekong, sampled during the dry and rainy season. Results represented the mean±SEM of 3 independent farms. Values not sharing a common letter are significantly different ($p<0.05$).

4. Discussion

Plasma osmolality values measured in this study were comprised within the range reported by other authors in striped catfish and other catfishes siluridae, differing by less than 10% (**Figure 3 A**) (Eckert et al.,2001; Phuc et al.,2014). In the dry season, increase in salinity up to 9 ppt elevated plasma osmolality to more than 280 mosm. On the contrary, gill Na^+K^+ ATPase activity slightly decreased with the proximity to the estuary. Similar responses to salinity have been described in channel catfish. Indeed, in this latter species, plasma osmolality increased during hyperosmotic stress but not the gill Na^+K^+ ATPase activity (Eckert et al.,2001). Channel catfish then compensated by producing reduced amount of concentrated urine (Norton & Davis, 1977). In our study, gill Na^+K^+ ATPase suddenly dropped by two during the rainy season (**Figure 4**), which may be induced by elevated suspended solids and particle uptake by gill epithelial cells. In aquatic animals, gills are generally considered as the primary target for suspended solids (Waters, 1995) In gills of salmonids (*Oncorhynchus spp.*), exposure to suspended solids induced important cytotoxic effects including physical damages of gills and oxidative stress (Martens and Servizi, 1993; Lake and Hinch, 1999; Michel et al.,2014).
Red and white blood cell abundances were similar to those obtained by Hang et al.,(2013) in the same species. Abundance of thrombocytes and monocytes in blood circulation of catfish raised in tidal sites, especially during the rainy season (**Table 2**). These results may indicate that these cells may increase with elevated suspended solids induced by monsoon runoff and coastal upwellings. Impact of long term exposure to suspended solids in blood composition has been poorly investigated. Similarly to our results, in the Gulf of Mexico, the EMAP (Environmental Monitoring and Assessment Program) demonstrated a correlation between the occurrence of macrophage aggregates and high organics load in several estuarine catfish species (i.e. sea catfish *Arius felis* and *Bagre marinus*, bullhead catfish *Ameirus nebulosus* and blue catfish *Ictalurus furcatus*) (Fournie et al.,2001). On the opposite, acute exposure of coho salmon *Oncorhynchus kisutch* to angular and round sediments above 40 g L^{-1} decreased leucocrit (Lake and Hinch, 1999).

Plasma lysozyme activity values measured in the dry season in upstream farms agree with those of Sirimanapong et al.,(2014), and differ by less than 10%. In our study, lysozyme activity increased in the rainy season as well as in tidal sites. (**Figure 5 B**). Salinity is known to increase the lysozyme activity in plasma of rainbow trout (Yada et al.,2001), brown trout (Marc et al.,1995), Nile tilapia (Dominguez et al.,2005), Mozambique tilapia (Jiang et al.,2008) and striped catfish (pers.com.). Then, gradual increase in salinity in the dry season may be responsible for elevated plasma lysozyme activities in tidal sites. In the rainy season, lysozyme activity may be enhanced by gradual increase in suspended solids, similarly to Nile Tilapia (Dominguez et al., 2005). Lysozyme activities in kidney were 50-fold lower than in plasma and did not differ among the study sites (**Figure 5 C**). In fish, lysozyme response to environmental variables is known to be tissue-specific. In channel catfish, 4 genes coding for lysozyme have been described (1 c-type gene and 3 g-type genes) and are differentially expressed depending on tissue and type of stressors (Wang et al., 2013). In this study, plasma and kidney complement activities were two to four fold superior to plasma complement activity measured in control fish in laboratory conditions (Hang et al.,2013; Sirimanapong et al.,2014) (**Figure 5 D**). To our knowledge, the effects of environmental factors on complement activity have been poorly investigated. In kidney, lower values have been measured in tidal sites during the dry season, suggesting a potential inhibitor effect of saline water on the alternative complement pathway in the striped catfish. In turbot *Scophthalmus maximus* L. reared in four salinity levels and challenged with *Vibrio anguillarum*, alternative complement activity in plasma decreased with increasing salinities (Zhang et al.,2011). The effects of particulate matter on ACH50 in aquatic organisms have not been investigated yet. Significantly higher values during the rainy season

suggest that the basal activity of alternative complement pathway might be slightly enhanced by elevated suspended solids.

Other seasonal and regional confounding factors may affect the immune parameters of striped catfish sampled from Mekong Delta fish farms. Disease outbreaks (Phan et al., 2009; Son et al.,2010) as well as higher coliform densities (Isobé et al.,2003) during the wet season suggest that water level change and sediment load should be accompanied with higher microorganism load. Therefore, it may be interesting to investigate the microorganism load during the wet season and saline intrusions. Therefore, it may be interesting to investigate the microorganism load during the wet season and saline intrusions. In addition, higher concentration of persistent organic pollutants such as polychlorinated biphenyl (PCB) and dichlorodiphenyltrichloroethane have been detected in sediments in the rainy season as well as in catfish farms near Can Tho city compared with downstream remote area (Minh et al.,2007). Furthermore, during the rainy season, algal blooms (i.e. *Phaeocystis globosa*) favoured by coastal upwellings and high river runoff were recorded in coastal waters of the Delta (Doan-Nhu et al.,2010; Dippner et al.,2011). This may significantly affect key physicochemical variables in catfish ponds (e.g. pH and oxygen level) and might interfere with immune or osmoregulatory factors, particularly the gill ion exchange mechanisms (Nieminen et al.,1982; Wang et al.,2002).

Most studies focus on specific environmental factors under controlled conditions rather than complex environmental changes. Indeed, the natural complexity of the environment generates a pattern of responses and it may be extremely difficult to establish specific causal relations. Nevertheless, a better understanding of how environmental factors affect fish physiology and immunity in aquaculture is of critical importance to predict changes and deleterious effects in health and disease resistance, and this is particularly true for species of high commercial importance as the striped catfish.

5. Conclusions

In the Mekong Delta, seasonal environmental conditions as well as the proximity to the estuary influence osmoregulation and basal immunity of the striped catfish. Special attention should be given to the high fluctuations of the Mekong River discharge associated with higher suspended solids load during the rainy season. During the dry season, salinity intrusion and modifications in water circulation may elevate the salinity into downstream catfish ponds and modify the integrity of key innate immune system components.

Acknowledgments: We are grateful to Prof. Nguyen Thanh Phuong for his welcome in Can Tho University. Thanks are due to FRIA (Fonds de la Recherche dans l'Industrie et l'Agriculture, Wallonia-Brussels Federation) for providing the PhD grant to M.S. and ARES-CCD (Commission of Interuniversity Cooperation for Development) for partially supporting the sampling costs of this study.

References

Bosma, R.H., Hanh, C., Potting, J. Environmental impact assessment of the Pangasius sector in the Mekong delta. Agriculture, nature and food quality. Report from Wageningen university, 2009, 1-58.

Choi, H.S., Kim, J., Cha, Y., Kim, C. A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. *J. Immunoassay Immunochem.,* 2006, *2,* 31-44.

Chou, H.Y., Peng, T.Y., Chang, S.J., Hsu, Y.L., Wu, J.L. Effect of heavy metal stressors and salinity shock on the susceptibility of grouper (*Epinephelus* sp.) to infectious pancreatic necrosis virus. *Virus Res.* 1999, *63,* 121-129.

De Silva,S., Phuong, N.T. Striped catfish farming in the Mekong Delta, Vietnam, a tumultuous path to a global success. *Rev. Aquacult.,* 2011, *3,* 45-73.

Dippner, J.W., Nguyen-Ngoc, L., Doan-Nhu, H., Subramaniam, A. A model for the prediction of harmful algae blooms in the Vietnamese upwelling area. Harmful Algae, 2011, *10,* 606-611.

Doan-Nhu, H., Nguyen-Ngoc, L., Dippner, J.W. Development of *Phaeocystis globosa* blooms in the upwelling water of the South Central Coast of Vietnam. *J. Marine Syst.,* 2010, *83,* 253-261.

Dominguez, M., Takemura, A., Tsuchiya, M. Effects of changes in environmental factors on the non –specific immune response of Nile tilapia, *Oreochromis niloticus*. *Aquac. Res. 36,* 391-397.

Eckert, S.M., Yada, T., Shepherd, B.S., Stetson, M., Hirano, T., Grau, E.G. Hormonal control of osmoregulation in the channel catfish *Ictalurus punctatus. Gen. Comp. Endocr.,* 2001, *122,* 270–286.

Ellis, A. Lysozyme activity. In: Stolen TC, Fletcher PD, Anderson BS, Roberson BS, Muiswinkel WB (ed) Technique in Fish Immunology, New York, 2001, 101-103.

FAO – Food and Agriculture Organization of the United Nations. The state of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations, Rome, 2014, 1-143.

Fournie J.W., Summers, J.K., Courtney, L.A., Engle, V.D., Blazer, V.S. Utility of splenic macrophage aggregates as an indicator of fish exposure to degraded environments. *J Aquat Anim. Health*, 2001, *13,* 105–116.

GRBC - The Global Runoff Data Centre. Online data from MRC flood forecasting. Global Runoff Data Centre, 56068 Koblenz, Germany, 2015.

Hang, B., Milla, S., Gillardin, V., Phuong, N., Kestemont, P. In *vivo* effect of *Esterichia coli* lipopolysaccharide on regulation of immune response and protein expression in striped catfish (*Pangasianodon hypophthalmus*). *Fish Shellfish Immun*., 2013, *34,* 339-347.

Intergovernmental Panel on Climate Changes. The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, IPPC, 2013.

Isobe, K.O., Tarao, M., Chiem, N., Minh, L.Y., Takada, H. Effect of environmental factors on the relationship between concentrations of coprostanol and fecal indicator bacteria in tropical (Mekong Delta) and Temperate (Tokyo) freshwater. *Appl. Environ. Microbiol.,* 2003, *70,* 814-821.

Jiang, I., Khumar, V., Lee, D., Weng, C. Acute osmotic stress affects tilapia (*Oreochromis mossambicus*) innate immune responses. *Fish Shellfish Immun*., 2008, *25,* 841-846.

Lake, R., Hinch, S.G. Acute effects of suspended sediment angularity on juvenile coho salmon (*Oncorhynchus kisutch*). *Can. J. Fish Aquatic. Sci*., 1999, *56,* 862-867.

Lefevre, S., Huong, D., Ha, N., Wang, T., Phuong, N.T., Bayley, M. A telemetry study of swimming depth and oxygen level in a Pangasius pond in the Mekong Delta. *Aquaculture,* 2011a, *315,* 410-413.

Lefevre, S., Jensen, F., Huong, D., Wang, T., Phuong, N., Bayley, M. Effects of nitrite exposure on functional haemoglobin levels, bimodal respiration, an swimming performance of the facultative air breather fish *Pangasianodon hypophthalmus*. *Aquat. Toxicol*., 2011, *104,* 86-93.

Lefevre, S., Huong, D., Wang, T., Phuong, N.T., Bayley, M. Hypoxia tolerance and partitioning of bimodal respiration in the striped catfish (*Pangasianodon hypophthalmus*). *Comp. Biochem. Phys. A*, 2011c, *158,* 207 – 214.

Magnadottir, B. Innate immunity of fish. *Fish Shellfish Immunol*., 2006, *20,* 137-151.

Marc, A.M., Quentel, C., Severe, A., Lebail, P.Y., Boeuf, G. Changes in some endocrinological and non-specific immunological parameters during seawater exposure in the brown trout. *J. Fish Biol.,* 1995, *46(6),* 1065-1081.

Martens, D.W., Servizi, J.A. Suspended Sediment Particles inside Gills and Spleens of Juvenile Pacific Salmon (*Oncorhynchus* spp.). *Can. J. Fish Aquat. Sci*., 50(3): 586-590.

Mc Cormick SD (1993) Methods for nonlethal gill biopsy and measurement of NaK-ATPase activity. *Can. J. Fish Aquatic Sci*., 1993, *50,* 656-658.

Mekong River Commission MRC. State of the Basin Report 2010. Mekong River Commission, Vientiane, 2010, pp 123.

Michel, C., Herzog, S., de Capitani, C., Burkhardt-Holm, P., Pietsch, C. Natural mineral particles are cytotoxic to rainbow trout gill epithelial cells in vitro. *Plos One*, 2014*, 9(7),* 1-11.

Minh, N., Minh, T.B., Kajiwara, N., Kunisue, T., Iwata, H., Viet, P.H., Tu, N.P., Tuyen, B., Tanabe, S. Pollution sources and occurrences of selected persistent pollutants (POPs) in sediments of the Mekong River delta, South Vietnam. *Chemosphere*, 2007, *67,* 1794-1801.

Nieminen, M., Korhonen, I., Laitinen, M. Effect on pH on the gill ATPase activity and blood composition of whitefish (*Coregonus peled*) and trout (*Salmo trutta fario*). *Comp Biochem Phys A,* 1982, *72(4*), 637-642.

Norton, V., Davis, K. Effect of abrupt change in the salinity of the environmental on plasma electrolytes, urine, volume, volume and electrolytic excretion in channel catfish, *Ictalurus punctatus*. *Comp Biochem Phys. A*, 1977*, 56*, 425-431.

Phan, L., Buy, T., Nguyen, T., Gooley, G., Ingram, B., Nguyen, H., Silva, S. Current status of farming practices of striped catfish, *Pangasianodon hypophthalmus*, in the Mekong Delta, Vietnam. *Aquaculture,* 2009, 296, 227-236.

Phuc, T., Huong, D., Mather, P., Hurwood, D.A. Experimental assessment of the effects of sublethal salinities on growth performance and stress in cultured tra catfish (*Pangasianodon hypophthalmus*). *Fish Physiol Biochem*, 2014, *40(6*), 1839-1848.

Rook, G., Steele, J., Umar, S., Dockrell, H. A simple method for the solubilisation of reduced NBT and its use as a colorimetric assay for activation of human macrophages by δ-interferon. *J Immunol Methods*, 1985, *82,* 161-167.

Secombes, C.J., Fletcher, T.C. The role of phagocytes in the protective mechanisms of fish. *Annu. Rev. Fish Dis*. 1992, 53-71.

Sirimanapong, W., Thompson, K., Kledmanee, K., Thaijongrak, P., Collet, B., Adams, A. Optimisation and standardisation of functional immune assays for striped catfish (*Pangasianodon hypophthalmus*) to compare their immune response to live and heat killed *Aeromonas hydrophila* as models of infection and vaccination. *Fish Shellfish Immun*., 2014, *40,* 374-383.

SIWRR-The Southern Institute of Water Resources Research. Saline intrusion in the Mekong Delta. http://www.tongcucthuyloi.gov.vn/, *accessed on March 24, 206.*

Son, V., Phuong, N., Hai, T.N., Yakupitiyage, A. Production and economic efficiencies of intensive black tiger prawn (*Penaeus monodon*) culture during different cropping seasons in the Mekong Delta, Vietnam. *Aquacult Int.,* 2011, *19,* 555-566.

Sunyer, J.O., Tort, L. Natural haemolytic and bactericidal activities of sea bream *Sparus aurata* serum are effected by the alternative complement pathway. *Vet Immunol Immunop*., 1995, *45,* 333-345.

Thanh, N.M., Jung, H., Lyons, R.E., Chand, V., Tuan, N.V., Thu, V., Mather, P. A transcriptomic analysis of the striped catfish (*Pangasianodon hypophthalmus*) in response to

salinity adaptation: de *novo* assembly, gene annotation and marker discovery. *Comp. Biochem. Physiol. D,* 2014, *10,* 52-63.

Uribe, C., Folch, H., Enriquez, R., Moran, G. Innate and adaptive immunity in teleost fish: a review. *Vet. Med.,* 2011, *56,* 486-503.

VASEP-Vietnam Association of Seafood Exporters and Producers. Pangasius export statistics. VASEP, Vietnam, 2015.

Vázquez, G., Guerrero, G. Characterization of blood cells and hematological parameters in *Cichlasoma dimerus* (Teleostei, Perciformes). *Tissue Cell.,* 2011, *39,* 151–160.

Wang, W., Wang, L., Chen, L., Liu, Y., Sun, R.Y. Effects of pH on survival, phosphorus concentration, adenylate energy charge and NaK ATPase activities of *Penaeus chinensis* Obsbeck juveniles. *Aquat Toxicol.*, 2002, *60,* 75-83.

Wang, R., Feng, J., Li, C., Liu, S., Zhang, Y., Liu, Z. Four lysozymes in catfish are drastically but differentially induced after bacterial infection. *Fish Shellfish Immun*., 2013, *35*, 136-145.

Waters, T.F. Sediment in streams: sources, biological effects and control. Am. *Fish Soc.Monogr.*, 1995, *126(6),* 1048-1051.

Yada, T., Azuma, T., Takaji, Y. Stimulation of non-specific immune functions in seawater-acclimated rainbow trout *Onchorynchus mykiss*, with reference to the role of growth hormone. *Comp. Biochem. Phys. B,* 2011, *129,* 695-701.

Zhang, Y., Mai, K., Ma, H., Ai, Q., Zhang, W., Xu, W. Rearing in intermediate salinity enhances immunity and disease-resistance of turbot *Scophthalmus maximus* L. *Acta Oceanol Sin.*, 2011, *30,* 122-128.

Additional comment: As mentioned in the discussion, the work does not provide data on the diversity and abundance of the bacterial load or the microorganism communities in the waters analysed. Moreover, the suspended solid contents and/or the presence of possible contaminants in the water is also unknown. Therefore, the supposition that immune factors were mainly driven by the suspended solid contents in this study, as suggested in the conclusion, is only a hypothesis.

Chapter 3

Chronic hyperosmotic stress interferes with immune homeostasis in striped catfish (*Pangasianodon hypophthalmus*, S.) and leads to excessive inflammatory response during bacterial infection.

Schmitz, M.; Douxfils, J.; Mandiki, S.N.M.; Morana, C.; Baekelandt, S.; Kestemont, K. Chronic hyperosmotic stress interferes with immune homeostasis in striped catfish (*Pangasianodon hypophthalmus*, S.) and leads to excessive inflammatory response during bacterial infection. *Fish Shellfish Immunol*., 2016, *55*, 550-8.

Abstract

Hyperosmotic stress has often been investigated from osmoregulation perspectives while the effects of such stress on the immune capacity remain largely unexplored. In this study, striped catfish were submitted to three salinity profiles (freshwater, low saline water, saline water) during 20 days, followed by infection with a virulent bacteria, *Edwardsiella ictaluri*, responsible for the enteric septicemia of catfish. Osmoregulatory (plasma osmolality, gill Na^+K^+ATPase), immune (blood cells, lysozyme activity, complement activity, respiratory burst) parameters and mortality rate were investigated. In addition, abundances of heat shock protein 70 and high mobility group box 1 were explored. With elevated salinity, plasma osmolality severely increased while gill Na^+K^+ATPase slightly increased. Salinity alone stimulated the number of granulocytes, lysozyme activity and respiratory burst but depleted the number of thrombocytes. Salinity in combination with infection stimulated the number of monocytes and ACH50. On the contrary, erythrocytes, hematocrit, heat shock protein 70 and high mobility group box 1 did not significantly vary with salinity profiles. Then, salinity induced earlier onset on mortalities after *E. ictaluri* inoculation whereas cumulative mortality reach 79.2%, 67.0% and 91.7% respectively in freshwater, low saline water and saline water. In conclusion, salinity stimulates several immune functions in striped catfish but prolonged exposure to excessive hyperosmotic condition may lead to excessive inflammatory response and death.

1. Introduction

The striped catfish (*Pangasianodon hypophthalmus*, Sauvage) is a potamodromous catfish endemic of the Mekong River Basin and the Chao Praya River in Thailand. Nowadays, striped catfish farms are the major inland aquaculture production in Southeast Asia. In 2014, worldwide striped catfish production reached 1.2 million of tons, which represent almost 1.7 billion US$ in the international trade (FAO, 2014). Vietnam is by far the largest striped catfish producer, exporting striped catfish to more than 80 countries, mainly in the European Union and United States (FAO, 2014). In Vietnam, the Mekong Delta accounts for more than 75% of the Vietnamese production with a total farming area of 5509 ha in 2011 (VASEP, 2015). Nevertheless, striped catfish industry in the Mekong Delta is currently facing many climatic challenges, particularly extensive salinity intrusion induced by the global climate changes. According to 4 RCP (representative concentrations pathways), ocean thermal expansion (30 to

55%) and glaciers melting (15 to 35%) will induce a global sea level rise comprised between 0.26 and 0.98 m by 2100. In the Mekong Delta, in 2014, saline water intrusions have already been observed up to 40 km far from the River Mouth in several agricultural provinces, thereby rising salinity level up to 9 ppt in catfish ponds (Schmitz et al., 2016a). The latter study also suggested that such salinity conditions may affect catfish hematological and immune status (Schmitz et al., 2016a).

Environmental salinity is an important parameter for aquatic organisms. Modification of salinity may be responsible for important biochemical and physiological troubles (Cuesta et al., 2005). Studies evaluating fish acclimation from hyposmotic to hyperosmotic environment (relative to plasma) have been mainly looking at changes in the osmoregulatory system while the effects of such stressors on the immune capacity remain largely unexplored. Gilthead seabream (*Sparus aurata*) acclimated to low saline water (6 ppt) showed lower peroxidase and alternative complement activity in plasma compared to fish acclimated to brackish (12 ppt) and saline water (38 ppt) (Cuesta et al., 2004). In Mozambique tilapia (*Oreochromis mossambicus*) , renal and plasma lysozyme activities increased 1 h and 24 h after transfer from freshwater to saline water (25 ppt) while respiratory burst increased in spleen and kidney as early as 8 h post transfer (Jiang et al., 2008). In rainbow trout (*Oncorhynchus mykiss*), 3-days acclimation to hyperosmotic water (12 and 29 ppt) resulted in elevated plasma lysozyme activity (Yada et al., 2001). Hypo- or hyperosmotic shocks in grouper fry (*Epinephelus sp.*) induced higher susceptibility to infectious pancreatic necrosis virus (Chou et al., 1999). Regarding adaptative immune function, a study on fish vaccination revealed that euryhaline sibling barramundi (*Lates calcarifer*) acclimated to seawater produced a higher adaptive mucosal antibody response than barramundi acclimated to freshwater while serum antibody response was not affected (Delamare-Debouteville et al., 2006).

In eukaryotes, Heat Shock Proteins (HSP) and High-Mobility Group B1 (HMGB1) are constitutive and highly conserved molecular chaperones for DNA and proteins (Abraham et al., 2000; Roberts et al., 2000; Tang et al., 2011). During cellular stress, HSPs and HMGB1 may be produced and released either actively or passively in the extracellular environment, in order to inhibit protein aggregation and to repair denatured proteins (Wang et al., 2004; Mayer et al., 2005; Norouzitallab et al., 2015). For immune defence, HMGB1 and HSPs (particularly HSP70 and 90) have key role in inflammation as well as in innate and adaptative immune function during bacterial or viral infection (Abraham et al., 2000; Baruah et al., 2014). In the extracellular environment, they act as damage-associated molecular patterns (DAMPs) and affect many aspects of the immune response, particularly through activation of cell surface innate immune receptor, typically Toll Like Receptor (Baruah et al., 2014). In fish, upregulation of HSP70 has been documented during biotic (i.e. infectious disease) and abiotic (e.g. osmotic, acidosis, heat, anoxia, toxins, protein degradation) stressors (Abraham et al., 2000). On the other hand, reports on HMGB1 are still limited and regulation of HMGB1 level by abiotic factors remains largely unknown. In red drum (*Sciaenops ocellatus*) and goldfish (*Carassius auratus*), bacterial infection elevated the abundance of HMGB1 transcript and protein from 12 to 48h post challenge (Chao et al., 2011; Xie et al., 2014).

Following sterile or microbial injury, inflammatory processes are essential for tissue and wound repair. However, prolonged exposure to the detrimental agents and continuous release of DAMPs (e.g. typically HSPs, HMGB1, S100 calcium-binding proteins and purine metabolites) may lead to excessive inflammation and severe immunopathological conditions that may end up with tissue damage and death (Chen & Nunez, 2010).

Understanding how salinity changes impact fish health and disease resistance is of critical importance, particularly for high commercial species such as the striped catfish. In the present study, we aimed to characterize the effects of hyperosmotic stressor on passive and induced innate immune defences of striped catfish. We hypothesized that prolonged exposure

to hyperosmotic conditions (10 and 20 ppt) might unbalance the immune homeostasis of healthy fish by inducing chronic inflammation. Key factors of the osmoregulatory, inflammatory and immune components have been analysed at different time points during salinity exposure and compared to values obtained under freshwater conditions. Then, we further hypothesized that chronic inflammation may result in an excessive inflammatory response following microbial challenge, leading to serious diseases status and death. To verify this hypothesis, fish were challenged with a virulent strain of *Edwardsiella ictaluri*, responsible for ESC (enteric septicaemia of catfish) disease (Hawke et al., 1981). Key parameters of the innate immune response, molecular chaperones (i.e. HSP 70 and HMGB1 protein) and cumulative mortality were compared between freshwater-held fish and salinity-challenged fish prior and after inoculation of the bacteria.

2. Materials and methods.

2.1 Fish and *in vivo* stress experiment

Investigations have been conducted according to the guidelines for animal use and care in compliance with Belgian and European regulation on animal welfare (ethical protocol n°KE 12/189). One week-old striped catfish (*Pangasianodon hypophthalmus*, Sauvage) were provided by the Nam Sai catfish farm (Ban Sang, Thailand). Juveniles were maintained in fish facilities in the University of Namur (Belgium) at 28°C under constant aeration and photoperiod (12L:12D) in recirculating aquaculture systems. Fish were daily fed *ad libitum* with commercial dry pellets (Troco Supreme 4.5 mm, Coppens, The Netherlands). After 3 months, fish (40-50 g) were equally divided into 3 experimental groups each including four 100 L tanks (30 fish/tank). Fish were acclimated to their new housing conditions during 10 days. The first group of fish was maintained in freshwater (0.4 ppt) during all the experiment. The second group of fish was exposed to low salinity water stressor namely to a gradual water salinity increase of 0.5 ppt per day during 20 days (0.4 to 10 ppt). The third group of fish was exposed to a higher saline stressor namely to a gradual salinity increase of 1 ppt per day during 20 days (0.4 to 20 ppt). Salinity was increased by adding marine salt (Ocean Fish, Prodac, Italy) mixed with tap water until day 20 and then remained stable during the following bacterial challenge. On day 20, fish were anaesthetized in tricaine methanesulfonate MS-222 (150 mg L^{-1}) and intraperitoneally injected with 0.025 ml g^{-1} fish of a bacterial solution (10^6 bacteria ml^{-1} ($LD50_{96h}$) of Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich)). Mortality was daily recorded during 10 days. Fish (6 fish/tank) were sampled at the following time points: days 0, 10, 20 and 23. On the sampling days, fish were randomly collected in the tanks with nets and anaesthetized in MS-222 (Sigma-Aldrich) (150 mg L^{-1}). Blood was collected by caudal vein puncture using a sterile 1 ml heparinized syringe within 5 min and euthanized by cervical dislocation. Blood was kept on ice until plasma was separated by centrifugation at 4°C (7 000 g, 10 min) and frozen at -80°C pending analyses. The whole kidney was rapidly collected by gentle scratching with tweezers along the vertebral column and immediately frozen in liquid nitrogen. Gill filaments from left arches 1-2 were taken out and immediately frozen in liquid nitrogen. Spleen was stored on ice in L-15 media until respiratory burst assay was carried out the same day. Physicochemical data were measured daily in the outlet pipe using a multiparameter probe (WTW, Multi 350i) : O_2:5.7±0.5 mg L^{-1}; pH: 8.4±0.24; temperature: 28.2±0.1 °C, N-NO_3^-: 3.55±2.27 mg L^{-1}; N-NO_2^-: 0.019±0.005 mg L^{-1}; N-NH_3^+: 0.19±0.25 mg L^{-1}. Measured salinities were closed to the expected values (±0.3 ppt) (**Figure 1**). The feed intake was measured daily.

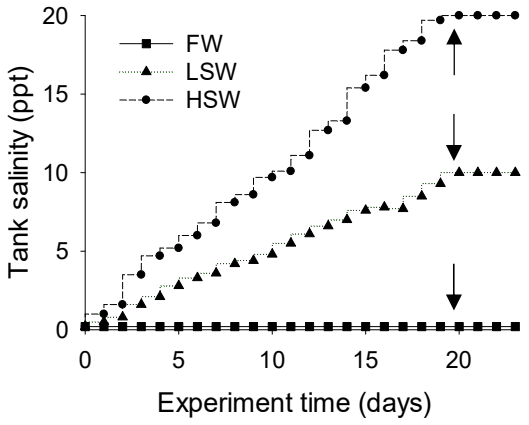


Figure 1: **Striped catfish salinity experiment regimens**. Striped catfish were exposed (or not) to increasing salinity during 20 days in three independent recirculating systems. FW: Freshwater; LSW: Low saline water (0-10 ppt); SW: saline water (0-20 ppt). The arrows represent bacteria inoculation at day 20.

2.2 Bacterial challenge

Culture of the bacterial strain
Virulent strain of *E. ictaluri* (TNA 015) was cultured on BHI (Brain Heart Infusion) agar (Sigma-Aldrich) at pH 7.4 and 28°C. Small round and transparent colonies appeared after 48 h of incubation. Colonies were checked for specific shape and Gram staining under light microscopy.
Bacteria count
Bacteria were first numbered in a reference bacteria solution with optical density of 0.1 at 590 nm. Serial exponential dilutions (100 to 10^{10}) of 100 µl of this reference bacteria solution were cultured in BHI agar during 48 h at 28°C and the colonies were counted. A method of confirmation using DNA fluorochrome 4'-6'-diamidino-2-phenylindole (DAPI) was used. Serial exponential dilutions (10^4 à 10^8) were incubated 1 h in DAPI, filtered and counted on black filters under fluorescent microscopy. Based on these bacteria counts, it has been estimated that a reference *E. ictaluri* solution of optical density 0.1 cultured in BHI contained approximately 10^9 bacteria ml^{-1} solution.
Lethal dose 50% (LD 50) 96h
In a preliminary experiment, exponential doses of bacteria suspended in HBSS were injected to fish using an inoculation volume of 0.025ml g^{-1} fish (4 tanks, n = 6 per tank) in order to estimate the LD 50_{96h}. The results indicated that an injection of 0.025 ml g^{-1} fish of a bacterial solution containing 10^6 CFU ml^{-1} induced 50 % mortality after 96h.
Confirmation of infection
Using Biolog biochemical identification systems, infection was confirmed on the kidney (100 % probability for *E. ictaluri*) by the CER group (Belgian reference laboratory for animal health, Centre d'Economie Rurale-CER, Aye, Belgium), a laboratory specialized in screening and diagnosis of fish viral, bacterial and parasitic pathologies.

2.3 Osmoregulatory parameters

Plasma osmolality
Plasma osmolality (100 µl) was measured with a micro-osmometer (Type 6, Löser Messtechnik, Germany) in duplicates according to the depression of freezing point compared to pure water.
Gill Na^+K^+ ATPase activity
Gill lysates were obtained by homogenizing (1:5) tissue in ice cold SEI buffer (Sucrose 0.25 M, EDTA 1 mM, Imidazole 50 mM - pH 7.4) containing a protease inhibitor cocktail (Sigma) for 2X30 s using a sterile potter homogenizer. Main debris were removed by 2 successive centrifugations at 10 000 g during 5 min at 4°C. An aliquot (50 µl) was used to measure the Na^+K^+ ATPase activity according to the method of Mc Cormick (1995). One unit of Na^+K^+ ATPase activity represents the consumption of 1 µmole NADH min^{-1} ml^{-1}. Analyses were performed in duplicates.

2.4 Hematology

Blood cell populations
Blood cells populations were analysed by flow cytometry (Flow Activated Cell Sorter Calibur, Flow Cytometry System) according to Inoue et al.(2002) [22] (**Figure 2**). Briefly, 10 µl of fresh heparinized blood were mixed with 1950 µl of HBSS and 40 µl of fluorochrome $DiOC_6$ (3,3-dihexyloxacarbocyanine, Sigma-Aldrich, Germany) diluted 1:10 in ethanol. The tube was mixed gently and incubated at RT (room temperature) during 10 min. The FACS was calibrated with TrueCount Beads diluted in HBSS. Each blood cell population was identified by its typical location in a FL-1 v. SSC and FSC v. SSC according to Inoue et al. (2002) and Pierrard et al.(2012) (**Figure 2**). Five clusters were identified including erythrocytes, thrombocytes and lymphocytes, monocytes, eosinophils and heterophils. In order to validate this method, differential blood counts were previously done in 20 fish in light microscopy. Regarding to erythrocytes, blood was diluted 200 X in HBSS and erythrocytes were counted on Neubauer hemocytometer. Regarding to leukocytes, subpopulations were differentially counted on blood smear staining with May Grunwald Giemsa according to Vazquez et al. (2007). **Figure 3** shows blood cell populations identified in light microscopy. Flow cytometer analyses were in agreement with microscopic counts.

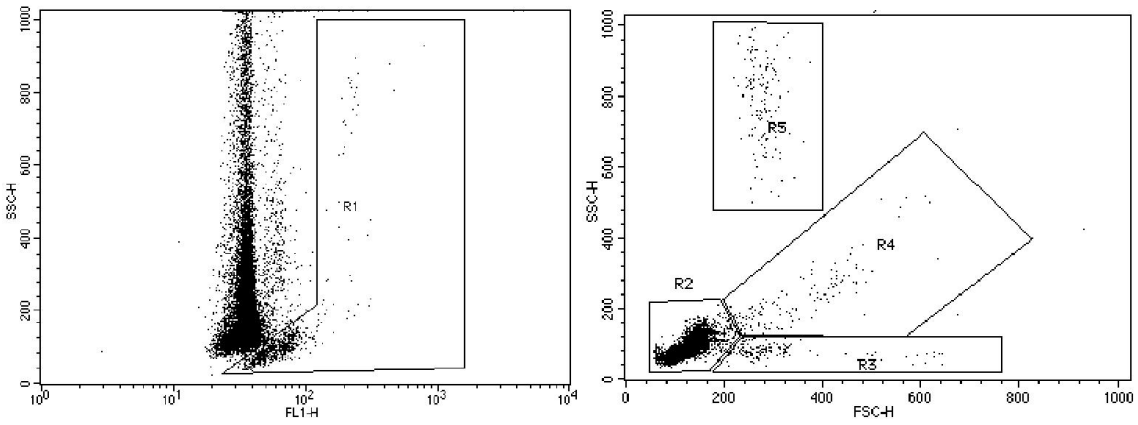


Figure 2: FACS analyses of blood cells of striped catfish stained with $DiOC_6$. (A) FL1 as a function of SSC, erythrocytes and isolation of leukocytes population (R1) (B) FSC as a function of SSC (R2: thrombocytes and lymphocytes; R3: monocytes; R4: eosinophils; R5: heterophils).

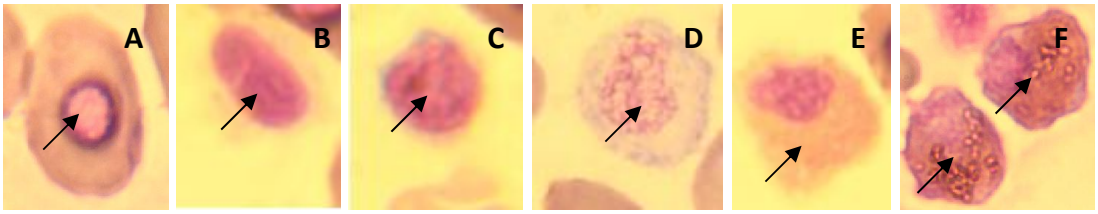


Figure 3: Blood smear of striped catfish stained by May Grunwald Giemsa. (A) erythrocytes; (B) thrombocyte; (C) lymphocyte; (D): monocyte; (E) eosinophil; (F) heterophils. Arrow indicates the corresponding cell.

Hematocrit
Fresh heparinized blood was centrifuged in microhematocrit tubes at 10 000 g during 5 min at RT.

2.5 Immune parameters

Plasma lysozyme assay
The lysozyme activity protocol was adapted from Ellis et al. (1990). Briefly, lysozyme activity assay was initiated by mixing 10 µl of kidney homogenate or 10 µl of plasma with 250 µl of lyophilized *Micrococcus lysodeikticus* (Sigma) suspension (0.6 mg ml^{-1} in phosphate buffer at pH 6.2). The difference in absorbance at 450 nm was monitored between 0 min and 15 min (linearity range). One unit of lysozyme activity represents the amount of lysozyme causing a 0.001 decrease in absorbance per minute. Samples were measured in duplicates.

Plasma alternative complement pathway
The alternative complement pathway was assayed in duplicates according to Sunyer and Tort (1995). Briefly, 10 µl of rabbit red blood cells suspension (RRBC, Biomerieux) suspended at 3% in veronal buffer (Biomerieux) were mixed with serial dilutions of plasma. After incubation for 2 h at 28°C, the samples were centrifuged (2000 g, 5 min, 4°C). A positive control sample (100% haemolysis) was obtained by adding 60 µl of distillate water to 10 µl of RRBC. A negative control sample was obtained by adding 60 µl of veronal buffer to 10µl of RRBC. The absorbance of supernatant was measured at 405 nm. The ACH 50 value is defined as the reciprocal of the plasma dilution which induces 50% haemolysis of RRBC.

Spleen respiratory burst
Spleen respiratory burst was adapted from Rook et al. (1985). Using the back of a syringe piston, spleen tissues were gently mashed with 1 ml of L-15 medium through a 100 µm nylon mesh grid settled at the bottom of a Petri dish. The cell suspension was washed twice and 100 µl of the final cell suspension were incubated in duplicates with 100 µl of nitroblue tetrazolium (1 mg ml^{-1} in PBS, pH 7.4) during 1h at RT. Then, samples were washed in PBS, methanol and finally air-dried at RT. The blue formazan in each tube was dissolved in 240 µl of KOH 2 M and 280 µl of N-dimethylformamide and absorbance was measured at 550 nm. Negative control samples were not incubated but directly brought to methanol fixation step. A standard curve was performed using serial dilutions of nitrobluetetrazolium directly dissolved in KOH 2M and N-dimethylformamide.

Kidney HSP70 and HMGB-1

Kidney lysates

Using Speed Mill Vac Bound Homogenizer, kidney lysates were obtained by homogenizing kidney tissue for 2X30s in the following buffer (1:3): Tris-HCl 50mM, NaCl 150 mM, SDS 0.1%, Triton X-100 0.1%, apopritin 0.001 mg ml^{-1}, pH 8. Lysates were then sonicated 3X10 s at 45 kHz and 5X1s at 65 kHz on ice and centrifuged at 10 000 g for 10 min to remove main debris. Total protein abundance in the samples was measured by Pierce method.

Blotting

Western blot analyses were performed to validate the specificity of the antibodies. So, 20 µg of kidney lysate proteins were mixed with dithiothreitol 0.5 M 0.1 % and NuPage Lithium Dodecyl Sulfate (1:3) and heated at 70°C for 7 min. Samples were then centrifuged at 13 000 g for 5 min to remove debris. Chemiluminescent ladder (ECL DualVue Marker, Amersham) and 20 µg of kidney proteins were separated on a 4-12% NuPage Novex Bis Tris gel during 1h30 at 100V and transferred onto a PVDF (polyvinylidene difluoride) membrane using an electrophoretic transfer system during 2h15 at 0.8 mA cm^{-2} (BioRad). Then the membrane was blocked overnight with PBST (PBS pH 7.4, 0.1% Tween-20) containing 5% skimmed milk at 4°C. The next day, the membrane was rinsed 2X5 min in PBST and probed with anti-HSP70 3A3 (ThermoScientific) 1:5000 diluted in 2% blocking buffer for 1h at RT under constant agitation. After washing 2X5 min with PBST, membrane was incubated with horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG antibody (GE Healthcare) 1:10 000 diluted in 2% blocking buffer for 1h at RT. Membrane was rinsed 2X10 min in PBST, 20 min in PBS and revealed with ECL Plus Western Blotting substrate (Thermo Scientific) in ChemiDoc MP Imaging System (BioRad). The same procedure was applied for HMGB-1 western blotting except that the PVDF membrane was probed with anti-HMGB-1 19N15F4 (ThermoScientific) 1:500 diluted in 2 % blocking buffer for 1h at RT as a primary antibody. Quantification was performed by dot blotting. Proteins extract (1 µl) was directly spotted to the wetted PVDF membrane and allowed to dry out during 30 min at RT. PVDF membrane was then transferred in a blocking solution (PBST, 5% skimmed milk) during 1h at RT and probed in the same manner than for western blotting. Dot quantification was done using Image J software.

2.6 Statistical analyses

Heterogeneity of variances was tested by Levene test and normality was checked by Shapiro-Wilk test. The changes in physiological, osmoregulatory and immune parameters were analysed by two-way analysis of variance ANOVA followed with pairwise multiple comparisons procedures by Scheffe test ($p<0.05$) in SigmaStat. Data are represented as the mean ± SD and tanks were used as the statistical unit (n = 4).

3. Results

3.1 Growth performance

The feed intake was significantly higher in fish held in freshwater (23.6±2.2 g $fish^{-1}$) compared to fish held in low saline (17.3±1.3 g $fish^{-1}$) and saline (18.2±2.5 g $fish^{-1}$) water. The weight gain ((final weight – initial weight) / initial weight * 100) and the specific growth rate (ln (final weight) – ln (initial weight)/ time*100) did not significantly vary between groups and averaged respectively 25.7±1.2% and 1.1±0.03%. Fulton's coefficient (K) ($100*weight/length^{2.88}$) was calculated on each sampling day (days 0, 10, 20 and 23) and averaged 1.04±0.05. K was significantly lower on day 23 compared with day 0, 10 and 20 ($p<0.001$).

3.2 Autopsy

Autopsies were performed on day 20 (n=6 fish per treatment) in collaboration with the CER laboratory. In the group exposed to saline water (0-20 ppt), 100 % of the fish were suffering from high congestions on fins and tail as well as on the membranous flap of skin of the opercula (**Table 1**). Only 15 % of the fish were suffering from congestion of barbels and abdomen. No symptoms were observed on the skin mucosal fluidity neither on gill integrity (fluidity, lamellar integrity and vascularisation). Dissection of mouth and ocular structures revealed no macroscopic damages or lesions. No ulcers or lesions were observed on the organs. During infection, all fish showed typical clinical signs of ESC (Enteric Septicemia of Catfish) including decrease in gill vascularization, abdominal septicaemia, high congestion of eyes, fins and tail, white gills and nodular round lesions (1-3 mm) on kidney, spleen and, to a smaller extent, on liver.

Table 1: Autopsy of striped catfish exposed to increasing salinity and macroscopic symptoms of ESC disease after 20 days of exposure to salinity increase (n=6 fish/treatment).

	FW	LSW	SW	Infected
Congestions of fins and tail	-	-	+	++
Congestion of opercula	-	-	+	++
Congestion of barbels and eyes	-	-	+	++
Diffuse abdominal congestion	-	-	+	++
Mucosal fluidity (skin)	+	+	+	+
Gill integrity (mucus, vascularisation)	+	+	+	-
Cutaneous petechial haemorrhages	-	-	-	+
Lesion or ulceration in organs	-	-	-	+
Abdominal septicaemia	-	-	-	+

Freshwater (FW), low saline water (LSW, 0-10 ppt) and saline water (SW, 0-20 ppt). -/+: absence/presence; +/++: comparative gravity of the lesions.

3.3 Osmoregulatory response

The osmoregulatory response of striped catfish to increasing salinity was investigated through plasma osmolality (**Figure 4 A**) and gill Na^+K^+ ATPase (**Figure 4 B**). In freshwater, plasma osmolality was comprised between 254 and 271 mosm. With increasing salinities, plasma osmolality gradually increased to reach 288±6 mosm at 10 ppt and 370±3 mosm at 20 ppt on day 20 ($p<0.001$). The bacterial infection did not induce significant changes in plasma osmolality. Prior to infection, gill Na^+K^+ ATPase activity was comprised between 0.27 and 0.75 U mg^{-1} gill min^{-1} in freshwater and low saline water respectively. At 20 ppt on day 20, activity increased up to 1.68 ± 0.32 U mg^{-1} gill min^{-1} ($p<0.05$). During infection, gill Na^+K^+ ATPase activity significantly decreased from 0.69 U mg^{-1} gill min^{-1} in freshwater to 0.38 at 20 ppt ($p<0.001$).

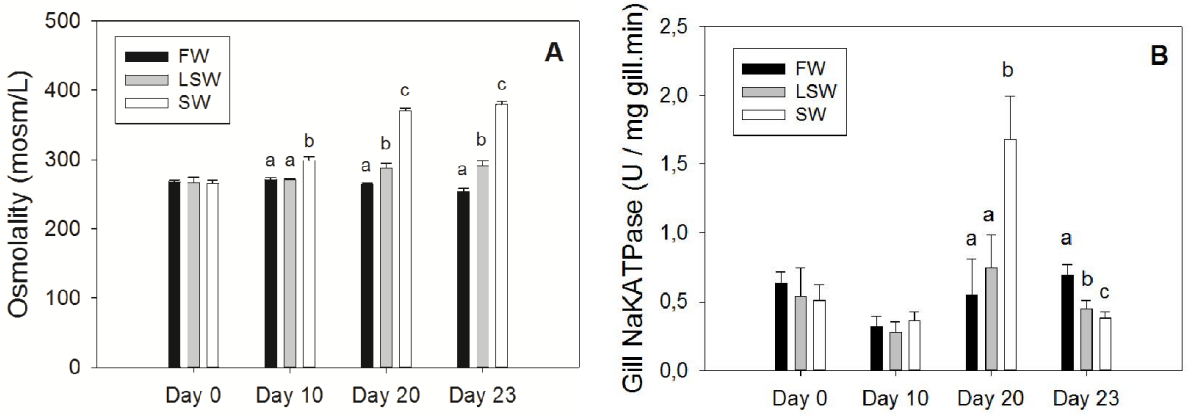


Figure 4: Osmoregulatory responses of striped catfish exposed (or not) to elevated salinity and experimentally infected with *Edwardsiella ictaluri*. Plasma osmolality (A) and gill Na^+K^+ ATPase activity (B) of striped catfish exposed to freshwater (FW, black), low saline water (LSW, grey) and saline water gradient (SW, white) during 20 days and 3 days post-infection (day 23). The values were presented as the mean ± SD with n=4 (4 tanks, 6 fish/tank). The statistical letters (a,b,c) indicate a significant change ($p<0.05$) between salinity treatments on the same sampling day.

3.4 Hematology

Salinity did not induce any significant effect on the number of erythrocytes and haematocrit (**Table 2**). Before infection, the abundance of heterophils increased with salinity, peaking at 29.3 million cells μl^{-1} blood at 20 ppt, which corresponds to a level 4 fold higher than in freshwater and low saline water ($p<0.01$) (**Table 2**). During infection, fish also exhibited significant changes in their blood cell populations (**Table 2**). The number of thrombocytes / lymphocytes significantly decreased by one third on day 23 in all groups compared with day 0, 10 and 20 ($p<0.001$). Moreover, increase in salinity also significantly lowered the number of thrombocytes / lymphocytes from 124.8 million cells μl^{-1} blood in freshwater to 82.53 million cells μl^{-1} blood in saline water ($p<0.01$). As the decrease is higher than the proportion of lymphocytes in the cluster, as suggested by microscopic stain, thrombocytes may be, at least partly, responsible for the drop of the cluster in cytometry. The number of eosinophils and heterophils significantly increased during infection (day 23) compared with day 0, 10 and 20 but was not significantly affected by salinity ($p<0.01$). During infection, the number of monocytes was enhanced with salinity by two fold to reach 30.7 million cells μl^{-1} blood.

Table 2: Hematology of striped catfish exposed (or not) to elevated salinity and experimentally infected with *Edwardsiella ictaluri*. The letters (a,b) indicate a significant change ($p<0.05$) between salinity treatment on a same sampling day.

		FW	LSW	SW
Erythrocytes (million cells µl^{-1} blood)	Day 0	3.84±0.20	4.10±0.20	4.09±0.40
	Day 10	3.85±0.09	3.81±0.12	4.15±0.28
	Day 20	4.08±0.18	3.87±0.36	4.36±0.15
	Day 23	4.37±0.13	4.13±0.27	4.6±0.22
Hematocrit (%)	Day 0	37.6±1.59	33.67±1.44	33.60±2.51
	Day 10	33.67±1.28	31.54±1.56	33.20±2.12
	Day 20	36.93±1.94	35.78±1.60	36.62±1.77
	Day 23	32.29±1.17	32.17±1.05	31.90±2.38
Thrombocytes and lymphocytes (thousand cells µl^{-1} blood)	Day 0	171.3±23	170.9±38.6	152.8±17.5
	Day 10	167.7±32.9	167.5±7.3	164.8±11.6
	Day 20	153.3±8.3	171.0±16.1	149.9±29.4
	Day 23	**124.8±15.2** ^{a}	**109.4±24.8** ^{ab}	**82.53±11.9** ^{b}
Monocytes (thousand cells µl^{-1} blood)	Day 0	18.1±13	7.3±1.9	10.2±8.2
	Day 10	7.3±5.4	13.1±9.1	6.8±4.6
	Day 20	39.3±19.2	16.8±11.1	14.6±6.0
	Day 23	**17.4±2.3** ^{ab}	**13.4±4.9** ^{a}	**30.7±8.9** ^{b}
Eosinophils (thousand cells µl^{-1} blood)	Day 0	7.6±2.6	9.0±1.9	8.9±8.4
	Day 10	7.8±1.9	7.5±1.4	5.2±0.7
	Day 20	7.6±3.4	5.4±1.4	6.9±0.7
	Day 23	8.7±0.9	11.0±7.2	12.9±1.6
Heterophils (thousand cells µl^{-1} blood)	Day 0	5.4±2.4	6.2±4.5	4.1±2.1
	Day 10	3.3±3.0	1.8±1.1	3.2±1.0
	Day 20	**7.2±3.7** ^{a}	**4.6±1.7** ^{a}	**29.3±6.3** ^{b}
	Day 23	17.8±4.4	11.3±9.0	11.9±5.1

Freshwater (FW), low saline water (LSW) and saline water (SW) during 20 days and 3 days post-infection (day 23). Values presented as the mean ± SD with n=4 (4 tanks, 6 fish/tank). The statistical letters (a,b) indicate a significant change ($p<0.05$) between salinity treatments.

3.5 Immune parameters

Salinity significantly enhanced plasma lysozyme activity, alternative complement pathway and spleen respiratory burst. The strongest significant effect was observed on plasma lysozyme activity which gradually but significantly increased with elevating salinity ($p<0.001$) (**Figure 5** A). On day 0, lysozyme activity varied between 194 and 219 U ml^{-1} plasma. On day 10, lysozyme activity peaked at 264 U ml^{-1} plasma in fish held in saline water compared to 217 U ml^{-1} plasma in fish held in low saline water and 199 U ml^{-1} plasma in fish kept in freshwater. On day 20, it reached 519 and 346 U ml^{-1} plasma respectively in fish of high and low saline water groups while it averaged 268 U ml^{-1} plasma in freshwater fish. During the bacterial challenge, lysozyme activity significantly increased by 3-fold compared to values observed in fish prior to infection ($p<0.001$) and the stimulating effect of salinity was maintained ($p<0.001$). Indeed, in freshwater condition lysozyme activity averaged 1268 U ml^{-1} plasma in freshwater fish, 1756 U ml^{-1} plasma in fish from low saline water and 1948 U ml^{-1} plasma those from saline water. No significant effects of salinity on the alternative complement activity, which varied between 7.3 and 12.5 U ml^{-1} plasma, were observed in fish prior to infection (**Figure 5 B**). However, ACH50 (Alternative Complement Hydrolysis 50%) significantly increased in infected fish (day 23), particularly in low saline water and saline water ($p<0.001$). ACH50 response was significantly higher during bacterial infection in fish from low saline water (12.5±5.0 U ml^{-1} plasma) compared to freshwater fish (22.8±4.2 U ml^{-1} plasma) ($p<0.01$).

Spleen respiratory burst significantly decreased up to 4 fold during the experiment in all groups ($p<0.001$) (**Figure 5 C**). On day 20, respiratory burst significantly increased up to 3.0±0.2 mg formazan g^{-1} spleen in fish held in saline water compared to those held in freshwater (2.2±0.2 mg formazan g^{-1} spleen) ($p<0.001$)

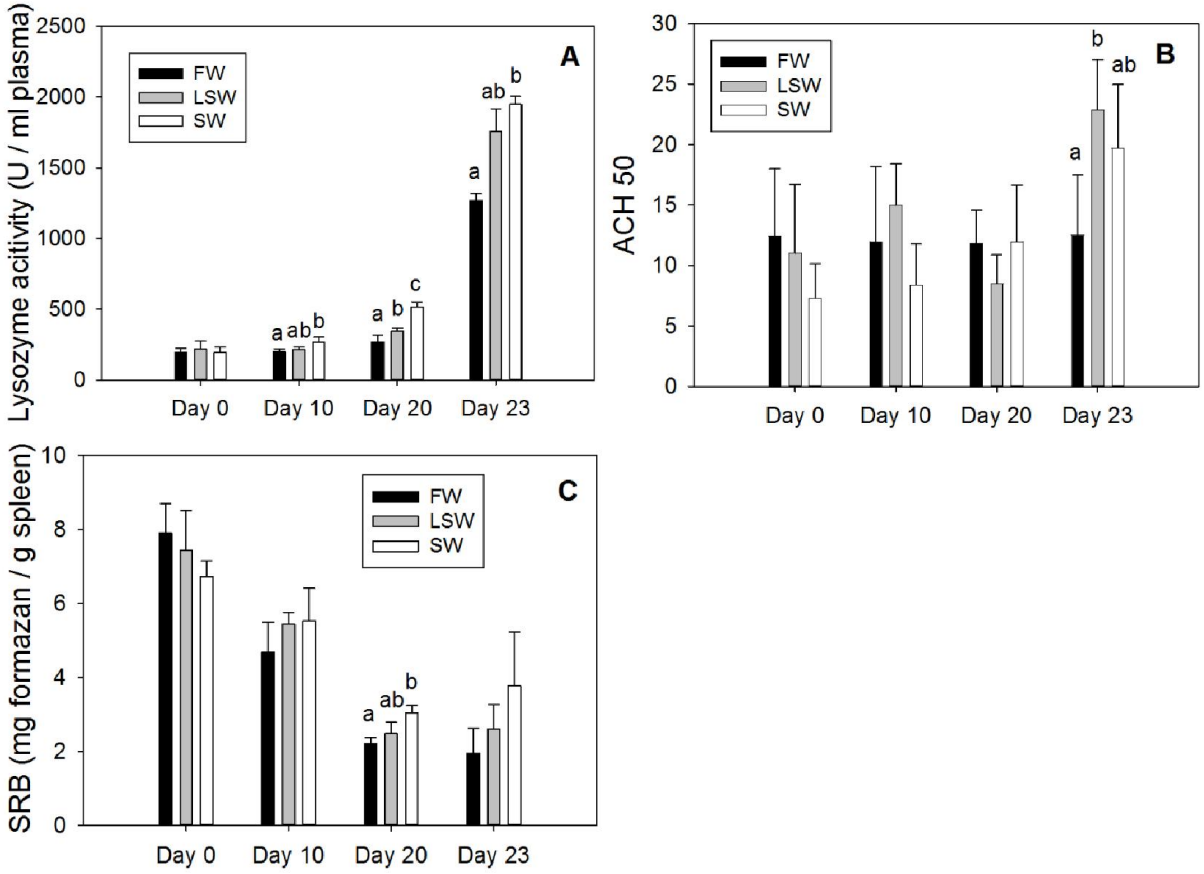


Figure 5: Immune response of striped catfish exposed (or not) to elevated salinity and experimentally infected with *Edwardsiella ictaluri*. Plasma lysozyme activity (A), alternative complement activity (B) and spleen respiratory burst (C) of striped catfish exposed to freshwater (FW, black), low saline water (LSW, grey) and saline water (SW, white) during 20 days and 3 days post-infection (day 23). Values were presented as the mean ± SD with n=4 (4 tanks, 6 fish/tank). The statistical letters (a,b,c) indicate a significant change ($p<0.05$) between salinity treatments on a same sampling day.

On the opposite, salinity did not induce any significant changes in the abundance of HSP70 and HMGB-1 in kidney. Relative to its abundance in freshwater fish from day 0, production of HSP70 varied from 0.46 and 1.63 fold on days 10, 20 and 23 (**Figure 6 A**). However, production of HMGB-1 was significantly higher in infected fish (day 23) compared to days 0, 10 and 20 ($p<0.001$). Relative production of HMGB-1 varied from 0.60 to 1.96 fold in fish prior to injection and increased 2.80 to 5.94 fold in infected fish, compared with the abundance of HMGB-1 in freshwater on day 0 (**Figure 6 B**).

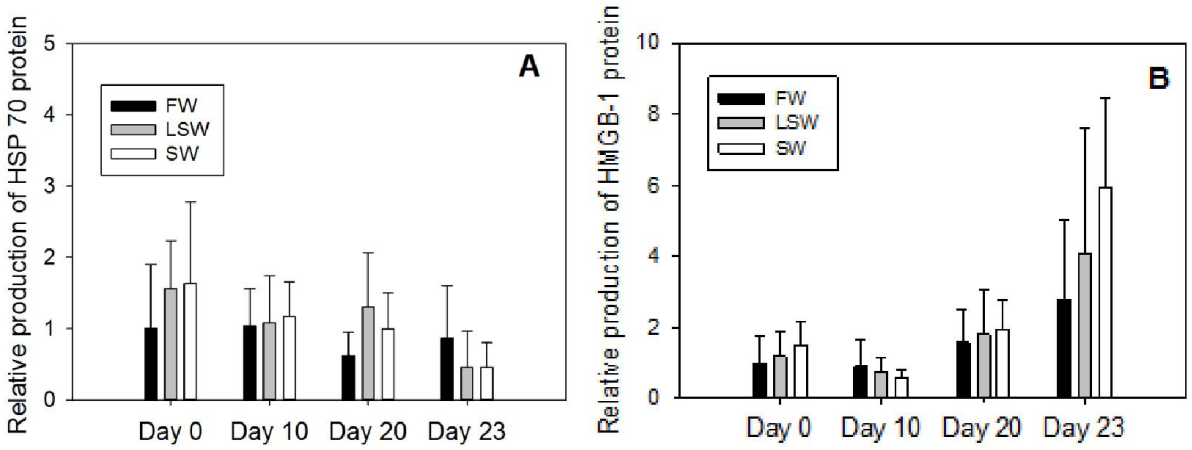


Figure 6: Chaperone response of striped catfish exposed (or not) to elevated salinity and experimentally infected with *Edwardsiella ictaluri*. Heat Shock Protein 70 (A) and High-Mobility Group protein B1 (B) of striped catfish exposed to freshwater (FW, black), low saline water (LSW, grey) and saline water (SW, white) during 20 days and 3 days post-infection (day 23). Values were presented as the mean ± SD with n=4 (4 tanks, 6 fish/tank).

3.6 Sensitivity to *Edwardsiella ictaluri.*

Figure 7 shows cumulative mortalities during 10 days after bacteria inoculation in the three salinity groups. At 72h post inoculation, mortalities were significantly higher in saline water (67±14%) compared to freshwater (0±0%) and saline water (13±16%) ($p<0.05$). At 96h post inoculation, mortalities begun in fish held in freshwater (42±10%) but were significantly lower than those recorded in saline water (75±10%) ($p<0.05$). On day 5, cumulative mortality was significantly higher in saline water (92±10%) compared to low saline water (67±14%). No additional mortalities were recorded from day 5 and up to day 10 post inoculation in the three salinity groups.

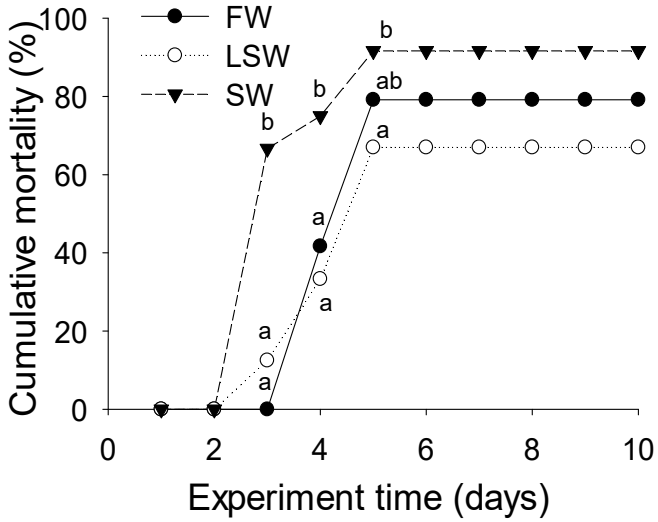


Figure 7: Cumulative mortality of striped catfish exposed (or not) to elevated salinity during 10 days after inoculation of *Edwardsiella ictaluri*. Mean cumulative mortality of striped catfish exposed to freshwater (FW), low saline water (LSW) and saline water (SW) during 10 days. The values were represented as the mean cumulative mortality (4 tanks, 6 fish/tank).

4. Discussion

Plasma osmolality values measured in this study were in the same range than those reported by other authors in striped catfish and other siluridae, differing by less than 10% (Eckert et al., 2001; Phuc et al., 2014). Increase in salinity was responsible for elevated blood plasma osmolality while gill Na^+K^+ ATPase activity only increased at 20 ppt. When striped catfish were submitted to low saline stress up to 10 ppt (270 mosm), plasma osmolality equilibrated with external salinity. However, higher salinity up to 20 ppt (540 mosm) induced a significant increase in plasma osmolality although to a lower level than that of the environment (370 mosm ± 3 mosm). This indicated that gill electrolyte clearance is insufficient to cope with the increased salt load. Similarly, seawater survival of channel catfish is limited due to the absence of efficient electrolyte excretion. Transfer of channel catfish from freshwater to seawater induced an elevation in plasma osmolality while the activity of functional renal glomeruli decreased (Norton & Davis, 1977; Eckert et al., 2001). In the current study, infection subsequent to salinity exposure lowered gill Na^+K^+ ATPase activity whereas plasma osmolarities remained unchanged. As live *E. ictaluri* gain access to blood circulation system across the gills, decrease in gill vascularisation is frequent in infected fish (including striped catfish in this study) and may be responsible for gill ion transport collapse (Yuasa et al., 2003; Shigen et al., 2009).

In saline water (20 ppt), striped catfish juveniles suffered from high congestions throughout the body. Prolonged high osmotic pressure in blood vessels may damage the endothelium and induce sterile inflammation. Particularly, high congestions have been observed on the opercular membrane, a membranous flap of skin located along the edge of the opercula that might play key role in resistance to hypoxia (Lefevre et al., 2011a). Whether increased osmotic pressure in the opercula may impair the resistance of striped catfish to hypoxia is unknown but should be investigated, as oxygen levels in typical Vietnamese aquacultures are inferior to 5 kPa (Lefevre et al., 2011b).

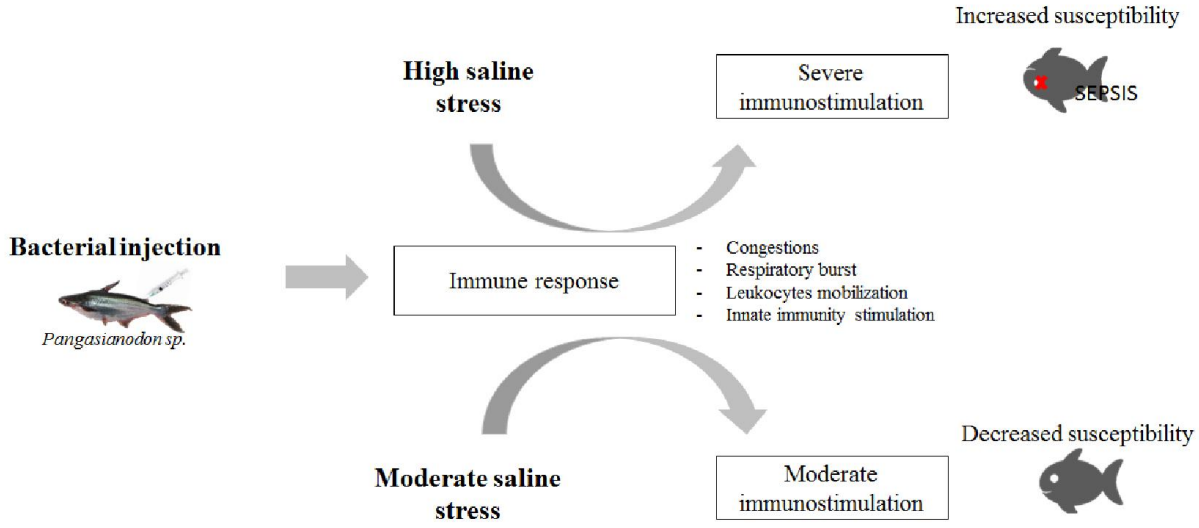
Blood composition and abundance of immune cells were modified by salinity in both healthy and infected fish. Prior to infection, the abundance of heterophils in the circulation of fish held in saline water increased by nearly 4 fold. During ESC, the abundance of macrophages increased by 2 fold in fish held in saline water while the abundance of thrombocytes was severely and gradually depleted. In this study, increase in monocytes and heterophils during hyperosmotic stress in healthy and infected fish respectively may indicate that the increased osmotic pressure is perceived as a threat to body integrity. In addition, the abundance of granulocytes (i.e. eosinophils and heterophils) increased in infected fish compared to the abundance before infection. Therefore, both stressors (infection and salinity) were responsible for accumulation of blood inflammatory cells including monocytes, eosinophils and heterophils. During prolonged cellular stress, it is known that persistence of such inflammatory cells may lead to excessive immune reaction and tissue damage (Chen & Nunez, 2010). On the contrary, salinity and infection had a synergic negative effect on the number of thrombocytes. In fish, decrease in the abundance of circulating thrombocytes is a frequent observation during pathogenic infection (Köllner et al., 2004; Fink et al., 2015) while the effects of salinity on thrombocytes have not been investigated yet. In infected fish, the increased blood osmotic pressure may activate the coagulation system in response to blood vessel damage and decrease the number of free circulating thrombocytes. Although their implication in hemostasis and wound healing is well-studied, the role of thrombocytes in immune functions is often neglected. Due to their extensively high number in the circulation, thrombocytes may play a major role in inflammatory processes and immunity. In fish, thrombocytes express a high number of immune-relevant genes involved in inflammation and antigen presentation, have the capacity

to phagocytose live bacteria and foreign particles and may have bactericidal activity (Köllner et al., 2004; Nagasawa et al., 2014).

In the present study, innate immune components were stimulated with increasing salinities or in combination with bacterial challenge. Plasma lysozyme activity of freshwater fish is in accordance with those of Sirimanapong et al. (2014) and differ by less than 10%. Salinity and infection rapidly and gradually stimulated lysozyme activity. In fish, higher lysozyme activities during both acute and chronic hyperosmotic stress have also been described in euryhaline species (Marc et al., 1995; Dominguez et al., 2005; Jiang et al., 2008). In this study, ACH50 values of freshwater fish are within the same range of values than those measured by Hang et al. (2013) on the same species. ACH 50 values increased during infection. In addition, a combination stimulatory effect of elevated salinity and infection was observed. In fish, variations of ACH50 during an osmotic stress have been poorly investigated. In gilthead seabream *Sparus aurata*, it has been shown that complement activities may increase or decrease when salinity increase, depending on acclimation time (Cuesta et al., 2005). The respiratory burst was within the same range of values those already observed for other species (Milla et al., 2010; Douxfils et al., 2011). A decrease in respiratory burst along the experiment has been observed and may be caused by insufficient acclimation time prior to the onset of the experiment. Our results suggest that salinity might stimulate spleen respiratory burst in striped catfish during hyperosmotic stress, similarly to the responses observed in several euryhaline species (Deane & Woo, 2004; Jiang et al., 2008), but such modulation was not markedly affected by bacterial challenge. During bacterial infection, excessive respiratory burst induced by the persistence of immune cells such as activated granulocytes and macrophages may lead to oxidative stress and tissue injury if not adequately countered by antioxidant activities (Chen & Nunez, 2010).

In our experiment, salinity stressor induced earlier onset of mortality in striped catfish during ESC. Moreover, cumulative mortalities after 10 days were significantly higher in fish held in saline water (92%) compared to fish held in low saline water (67%). In grouper fish, acute osmotic shock during 48h (33 ppt to 20 or 40 ppt) increased susceptibility to birnavirus from 10 to 90% (Chou et al., 1999). Therefore, high salinity stressor may increase sensitivity to ESC. At the opposite, low salinity water may have a protective effect in striped catfish by preventing bacteria multiplication.

In this study, HSP70 level remained unchanged with saline gradient alone the experiment. During acute or chronic hyperosmotic stress, increase in branchial HSP70 expression has been documented in several euryhaline species (Deane & Woo, 2004; Niu et al., 2008; Tine et al., 2010) while HSP70 modulation in other tissues remained unclear. In silver seabream acclimated to a large range of salinities, expression of HSP70 multigene family remained unchanged in kidney (Deane & Woo, 2004). Similarly to HSP70, no significant changes were observed in HMGB1 level during salinity exposure whereas HMGB1 level increased in infected fish compared to no infected fish. In addition, following infection, a non-significant increase was observed with salinity. In mammals, active secretion of HMGB1 mainly occurs when immunologically competent cells are exposed to pathogen or microbial associated molecular patterns (Anderson & Tracey, 2011; Lu et al., 2014). On the contrary, sterile injury did not induce HMGB1 production but only leaded to passive release of inactive HMGB1 (Anderson & Tracey, 2011; Lu et al., 2014). In the present study, it might be possible that sterile tissue damages induced by the hyperosmotic pressure did not induce active overproduction of HSP70 and HMGB1 but that passive extracellular release of HSP70 and HMGB1 occurred, making available some inflammatory mediators. Further, high standard deviation may reflect difference in HSP70 and HMGB1 response depending on individual fish susceptibility to the pathogen.



5. Conclusions

The present study demonstrated that salinity alone or salinity associated to infection increased some immune functions (i.e. lysozyme activity, complement activity, respiratory burst, abundance of monocytes and heterophils). Nevertheless, increase in immune factors does not necessarily indicate higher resistance to microbial disease, as suggested by the higher mortality rate in saline water. Chronic increase in the osmolality of body fluids may result in the persistence of sterile inflammatory processes such as granulocytes and macrophages accumulations, respiratory burst, release of inflammatory mediators and proteases. Therefore, it can be expected that such prolonged inflammatory response may lead to immune exhaustion and that the resulting tissue damages may disrupt basal immune homeostasis, thereby creating an unfavourable environment for efficient immune defence in case of pathogen contamination.

In conclusion, these novel results show the importance of the multi-stress approach in fish. To a larger extent, salt is commonly used as an antiseptic to prevent and treat microbial diseases in fish farms. In striped catfish ponds, production under moderate salinities has already been suggested in order to prevent microbial contamination. However, salt sterilization process targeting halophile pathogens should be managed carefully in order to avoid prolonged exposure of fish to hyperosmotic conditions which might be responsible for immune defence impairment.

Acknowledgements: We are grateful to the staff of CEFRA-Tihange (Ulg) and particularly to Christian Prignon for providing useful advices in catfish rearing and to Christian Didembourg in URBM (University of Namur) for his assistance during culture in bacterial strain and flow cytometer analyses. Thanks are due to FRIA (Fonds de la Recherche dans l'Industrie et l'Agriculture, Wallonia-Brussels Federation) and UNamur (Université de Namur) for providing a PhD grant to M.S.

References

Abraham, E., Arcaroli, J., Carmody, A., Wang, H., Tracey, K.J. HMG-1 as a mediator of acute lung inflammation. *J. Immuno*., 2000,*165,* 2950-2954.

Andersson U., Tracey, K. HMGB1 Is a Therapeutic Target for Sterile Inflammation and Infection. *Annu. Rev. Immunol*., 2011*, 29*, 139-162.

Baruah, K., Norouzitallab, P., Linayati, L., Sorgeloos, P., Bossier P. Reactive oxygen species generated by a heat shock protein (Hsp) inducing product contributes to Hsp70

production and Hsp70-mediated protective immunity in Artemia franciscana against pathogenic vibrios. *Dev. Comp. Immunol*., 2014, *46*, 470-479.

Chase, M., Wheeler, D., Lierl, K., Hughes, V., Wong, H., Page, K. Hsp72 induces inflammation and regulates cytokine production in airway epithelium through a TLR4 and NF kappa B dependent mechanism. *J. Immunol*., 2007, *170*, 6318-6324.

Chen, G.Y., Nunez, G. Sterile inflammation: sensing and reacting to damage. *Nature Rev.,* 2010, *10,* 26-837.

Chou, H., Peng, T., Chang, S., Hsu, Y., Wu, J.. Effect of heavy metal stressors and salinity shock on the susceptibility of grouper (*Epinephelus* sp.) to infectious pancreatic necrosis virus. *Virus Res*., 1999, 63, 121-129.

Cuesta, A., Laiz-Carrion, R., Martin del Rio, M., Meseguer, J., Mancera, J., Esteban, M. The salinity influences humoral immune parameters of gilthead seabream (*Sparus aurata*). *Fish Shellfish Immun.,* 2005, *18*, 255-261.

Deane, E.E., Woo, N.Y. Differential gene expression associated with euryhalinity in sea bream (*Sparus sarba*). *Am. J. Physiol. Integr. Comp. Physiol.,* 2004, *287*, 1054-1063.

Delamare-Deboutteville, J., Wood, D., Barnes, A.C.. Response and function of cutaneous mucosal and serum antibodies in barramundi (*Lates calcarifer*) acclimated in seawater and freshwater. *Fish Shellfish Immun*., 2006, *21,* 92-101.

Douxfils, J., Mathieu, C., Mandiki, S., Milla, S., Henrotte, E., Wang, N., Vandecan, M et al. Physiological and proteomic evidences that domestication process differentially modulates the immune status of juvenile Eurasian perch (Perca fluviatilis) under chronic confinement stress. *Fish Shellfish Immun*., 2011, *31*, 1113-1121.

Dominguez, M., Takeruma, A., Tsuchiya, M. Effects of changes in environmental factors on the non-specific immune response of Nile tilapia, *Oreochromis niloticus* L. Aquaculture Res., 2005, *36,* 391-397.

Eckert, S., Yada, T., Shepherd, B., Stetson, M., Hirano, T., Grau, E.G. Hormonal freshwater of osmoregulation in the channel catfish *Ictalurus punctatus. Gen. Comp. Endocr*., 2001, *122,* 270–286.

Ellis, A.E. Lysozyme activity. In: Stolen TC, Fletcher PD, Anderson BS, Roberson BS, Muiswinkel WB, editors. Technique in Fish Immunology. New York: SOS Publications; 1990: 101-103.

FAO – Food and Agriculture Organization of the United Nations. The state of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations. Rome, 2014.

Fink, I., Ribeiro, C., Forlenza, M., Taverne-Thiele, A., Rombout, J., Savelkoul, H., Wiegertjes, G. Immune relevant thrombocytes of common carp undergo parasite induced nitric oxide-mediated apoptosis. *Dev. Comp. Immunol*., 2015, *50,* 146-154.

Hang, D., Milla, S., Gillardin, V., Phuong, N., Kestemont, P. *In vivo* effects of *Escherichia coli* lipopolysaccharide on regulation of immune response and protein expression in striped catfish (*Pangasianodon hypophthalmus*). *Fish Shellfish Immun*., 2013, *34,* 339-347.

Hawke, J-P., McWhorther, A.C., Steigerwalt, A.G., Brenner, D.J. *Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicaemia of catfish. *Int. J. Syst. Bacteriol*., 1981, *31,* 396-400.

Inoue, T., Moritomo, T,. Tamura, Y., Mamiya, S,. Fujino, H., Nakanishi, T. A new method for fish leucocyte counting and partial differentiation by flow cytometry. *Fish Shellfish Immun.,* 2002, *13*, 379 – 390.

Jiang, I-F., Khumar, V-B., Lee, D-N., Weng, C-F. Acute osmotic stress affects tilapia (*Oreochromis mossambicus*) innate immune responses. *Fish Shellfish Immunol*., 2008, *25*, 41-846.

Köllner, B., Fisher, U., Rombout, J.H., Taverne-Thiele, J.J., Hansen, J.D. Potential involvement of rainbow trout thrombocytes in immune functions: a study using a panel of monoclonal antibodies and RT-PCR. *Dev. Comp. Immunol*., 2004, *28,* 1049-1062.

Lefevre, S., Huong, D.T., Wang, T., Phuong, N., Bayley, M. Hypoxia tolerance and partitioning of bimodal respiration in the striped catfish (*Pangasianodon hypophthalmus*). *Comp. Biochem. Phys. A*, 2011a, *158*, 207 – 214.

Lefevre, S., Huong, D.T.T., Ha, N., Wang, T., Phuong, N., Bayley, M. A telemetry study of swimming depth and oxygen level in a Pangasius pond in the Mekong Delta. *Aquaculture,* 2011b, *315*, 410-413.

Milla, S., Mathieu, C,. Wang, N., Lambert, S., Nadzialek, S., Massart, S., Henrotte, E., Douxfils, J., Melard, C., Mandiki, S., Kestemont, P. Spleen immune status is affected after acute handling stress but not regulated by cortisol in Eurasian perch, *Perca fluviatilis*. Fish

Lu, B., Wang, C., Wang, M., Li, W., Chen, F., Tracey, K.J., Wang, H. Molecular mechanism and therapeutic modulation of high mobility group box 1 release and action: an updated review. *Expert Rev. Clin. Immunol*., 2014*, 10*, 713-727.

Marc, A.M., Quentel, C., Severe, A., Le Bail, P., Boeuf, G. Changes in some endocrinological and non-specific immunological parameters during seawater exposure in the brown trout. *J. Fish Biol*., 1995, *46,* 1065-1081.

Mayer, M.P., Bukau, B. Hsp 70 chaperones: cellular function and molecular mechanism. *Cell. Mol. Life Sci.,* 2005, *62*, 670-684.

Mc Cormick, SD. Methods for nonlethal gill biopsy and measurement of NaK-ATPase activity. *Can. J. Fish. Aquat. Sci.,* 1993*, 50,* 656-658.

Nagasawa, T., Nagayasu, C., Rieger, A., Barreda, D., Somamoto, T., Nakao, M. Phagocytosis by thrombocytes is a conserved innate immune mechanism in lower vertebrates. *Front. Immunol.,* 2014, *5,* 1-11.

Niu, C.J., Rummer, J.L., Brauner, C., Schultz, P.M. Heat Shock Protein induced by a mild heat shock slightly moderates plasma osmolality upon salinity transfer in rainbow trout (*Onchorynchus mykiss*). *Comp. Biochem. Physiol*. C, 2008, *148*, 437-444.

Norouzitallab, P., Kartik, B., Muthappa, D.M., Bossier, P. Non lethal heat shock induces HSP70 and HMGB1 protein production sequentially to protect *Artemia franciscana* against *Vibrio campbelii*. *Fish Shellfish Immun*., 2015, *42*, 395-399.

Norton, V., Davis, K. Effect of abrupt change in the salinity of the environmental on plasma electrolytes, urine, volume, volume and electrolytic excretion in channel catfish, *Ictalurus punctatus. Comp. Biochem. Phys. A*, 1977, *56*, 425-431.

Phuc, T., Huong, D.T.T., Mather, P., Hurwood, D. Experimental assessment of the effects of sublethal salinities on growth performance and stress in cultured tra catfish (*Pangasianodon hypophthalmus*). *Fish Physiol. Biochem.,* 2014, *40*, 1839-1848.

Pierrard, M-A., Roland, K., Kestemont, P., Dieu, M., Raes, M., Silvestre, F. Fish peripheral blood mononuclear cells preparation for future monitoring applications. *Anal. Biochem.,* 2012, *426*, 153-165.

Roberts, R.J., Agius, C., Saliba, C., Bossier, P., Sung, Y.Y. Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. *J. Fish Dis.*, 2010, *33,* 789-801

Rook, G., Steele, J., Umar, S., Dockrell, H.M. A simple method for the solubilisation of reduced NBT and its use as a colorimetric assay for activation of human macrophages by δ-interferon. *J. Immunol. Methods*, 1981, *82*, 161-167.

Shigen, Y., Hua, L., Guo, Q., Zhongshi, L. First case of Edwardsiella ictaluri infection in China farmed yellow catfish *Pelteobagrus fulvidraco*. Aquaculture, 2009, *292,* 6-10.

Sirimanapong, W., Thompson, K., Kledmanee, K., Thaijongrak, P., Collet, B., Adams, A. Optimisation and standardisation of functional immune assays for striped catfish

(*Pangasianodon hypophthalmus*) to compare their immune response to live and heat killed *Aeromonas hydrophila* as models of infection and vaccination. *Fish Shellfish Immun*., 2014, *40*, 374-383.

Sunyer, J.O., Tort, L. Natural haemolytic and bactericidal activities of sea bream *Sparus aurata* serum are effected by the alternative complement pathway. *Veterinary Immunol. Immunop. ,* 1995, *45,* 333-345.

Tine, M., Bonhomme, F., McKenzie, D., Durand, J-D. Differential expression of the heat shock protein Hsp70 in natural populations of the tilapia, *Sarotherodon melanotheron*, acclimatised to a range of environmental salinities. *BMC Ecology,* 2010, *10*, 1-8.

Tang, D., Kang, R., Zeh, H., Lotze, M.T. High-mobility group box 1, oxidative stress,and disease. *Antioxid. Redox. Sign*., 2011, *14,* 11315-35

Vázquez, G.R., Guerrero, G.A. Characterization of blood cells and hematological parameters in *Cichlasoma dimerus* (Teleostei, Perciformes). *Tissue Cell*, 2007, *13*, 151–160.

Vietnam Association of Seafood Exporters and Producers (VASEP). Pangasius export statistics. VASEP 2015, Vietnam.

Wang, H., Liao, H., Ochani, M., Justiniani., M., Lin, X., Yang, L., Al-Abed, Y., Wang, H., Metz, E.J., Tracey K.J., Ukkoa L. Cholinergic agonists inhibiti HMGB1 release and improve survival in experimental sepsis. *Nat. Med.,* 2004*, 10*, 1216-1221.

Xie, J., Hodgkinson, J. W., Kovacevic, N., Belosevic, M. Identification and functional characterization of the goldfish (*Carassius auratus* L.) high mobility group box 1 (HMGB1) chromatin binding protein. *Dev. Comp. Immunol*., 2014, *44*, 245-53.

Yada, T., Azuma, T., Takaji, Y. Stimulation of non-specific immune functions in seawater-acclimated rainbow trout *Oncorhynchus mykiss*, with reference to the role of growth hormone. *Comp. Biochem. Phys*. B, 2001, 8, 695-701.

Yuasa, K., Kholidin, E., Panigoro, N., Hatai, K. First isolation of *Edwardsiella ictaluri* from cultured striped catfish *Pangasius hypophthalmus* in Indonesia*. Fish Pathol*., 2003, *38* 181-183.

Zhao, L., Hu, Y., Sun, J., Sun, L. The high mobility group box 1 protein of *Sciaenops ocellatus* is a secreted cytokine that stiumates macrophage activation*. Dev. Comp. Immunol*., 2011*, 35,* 1052-1058.

Chapter 4

Low saline stress enhanced physiological and immune pathways in striped catfish *Pangasianodon hypophthalmus* (Sauvage).

Abstract

In the Mekong Delta, striped catfish are faced to chronic hyperosmotic stress related to salinity intrusion induced by the global climate changes. In this study, striped catfish juveniles were submitted to a gradual hyperosmotic stress (up to 10 ppt) within 3 weeks, followed by an infection with a virulent strain of an intracellular bacteria, *Edwardsiella ictaluri*. Osmoregulatory parameters (gill NaK ATPase and plasma osmolality) were investigated. In addition, a label free quantitative proteomics workflow was performed on kidney. The flow consisted in initial global profiling of relative peptide abundances (by LC/MS) followed by identification (by MS/MS). Differentially expressed proteins were clustered in functional categories and visualized in KEGG pathway maps. The aim of the study was to highlight specific functional pathways modified during realistic and low hyperosmotic stress, particularly those involved in the immune response. In kidney proteome, 2483 proteins were identified, among which 400 proteins were differentially expressed. Hierarchical clustering analysis led to the isolation of two main clusters mainly driven by the infection status. Several pathways and functional categories, mostly related to energy metabolism, protein metabolism and processes, peroxisome, actin cytoskeleton rearrangement, immunity and detoxification were highlighted. In particular, the responsiveness of proteins involved in small GTPases and MAPK p38 signalling cascade, phagolysosome maturation and T cells regulation is discussed.

1. Introduction

Striped catfish (*Pangasianodon hypophthalmus*, Sauvage) industry has become by far the major inland aquaculture production in Southeast Asia, particularly in the Mekong Basin in Vietnam. In 2014, striped catfish production reached 1.2 million of tons and was exported to over than 151 countries, mainly in the United States and the European Union (FAO, 2014). Since 2004, several studies have pointed out the great concern regarding the effects of sea-level rise in the Mekong Basin on the agricultural development of the region (Nguyen et al., 2014). In South East Asia, long-term projections of sea-level rise predicts an annual increase in sea level by 2 to 4 mm per year (IPCC, 2013).

In aquatic organisms, several major evolutionally-conserved processes such as energetic metabolism, protein metabolism, lipid metabolism, redox homeostasis, ionic homeostasis and stress response have been pointed out by several authors in response to acute or chronic salinity stressors (Boutet et al., 2006; Kalujnaia et al., 2012; Kultz et al., 2013; Kumari et al., 2015). However, acclimation mechanisms from hyposmotic to hyperosmotic environment have been investigated mainly in terms of changes in osmoregulation while the effects of such osmotic stressor on the immune system remain largely unexplored. In teleost fish, chronic hyperosmolarity have been correlated with increase in abundance and activity of immune cells, lysozyme activity, complement activity, total IgM levels and higher mucosal antibody response (Yada et al., 2001; Cuesta et al., 2005; Delamare et al., 2006; Jiang et al., 2008). In mammals, a growing body of evidence suggests that chronic hyperosmotic stressors function as

inflammatory mediators, triggering pro-inflammatory cytokines release and innate immune cells inflammatory response (Schwartz et al., 2009; Brocker et al, 2012). Similarly, in fish, several studies demonstrated the implication of inflammatory proteins during hyperosmotic stress. Gill proteome of Mozambique tilapia *Oreochromis mossambicus* upregulated heat shock proteins and T-complex protein 1 members during short term exposure at 34 ppt (Kultz et al., 2013). Gill proteome of seawater-acclimated Japanese eels *Anguilla japonica* differentially expressed several immune proteins involved in the signalling of IL-6, IL-8, IL-9, HMGB-1 and iNOS (Lai et al., 2015). In addition, broad-nosed pipefish infected with *Vibrio* sp. lowered the expression of two key anti-inflammatory molecules (Granulocyte Colony Stimulating Factor and IL-10) after 3-days exposure to hyperosmotic conditions (6, 18 or 30 ppt) (Birrer et al., 2012). However, prolonged exposure to hyperosmotic stressor may lead to excessive inflammatory response and tissue damages (Chen & Nunez, 2010;).

Functional proteomics and transcriptomics technologies are becoming by far powerful tools to identify sets of genes and proteins involved in salinity adaptation. Particularly, the development of nano-flow liquid chromatography techniques associated with high-sensitivity spectrometry has enabled large-scale quantitation of proteins. The suitability of label free quantitation (LFQ) to characterize the phenotype of animal organ at a molecular level has already been debated in several independent studies (Cutillas et al 2007; Wang et al 2012). Quantitation of ion current intensity was used for relative quantitation of protein abundances in kidney tissue of fish raised in chronic hyperosmotic stressor compared to fish raised in freshwater. Then, the hyperosmotic stressor was combined with a bacterial infection. Kidney was chosen for our proteomic study as it lies at the crossroads of several major biological systems such as osmoregulation and immunity.

In this study, striped catfish juveniles (*Pangasianodon hypophthalmus*) were submitted to gradual hyperosmotic stressor during 20 days up to 10 ppt. The hyperosmotic stress was followed by an infection with a virulent bacterial strain of *Edwardsiella ictaluri*, responsible for the Enteric Septicemia of Catfish (ESC) (Hawke et al 1998), the major cause of mortality in catfish industry (USDA, 2011). Main osmoregulatory parameters (i.e. plasma osmolality and gill Na^+K^+ ATPase activity) were investigated. Using spectrometry-based label free proteomics, kidney proteome of salinity-exposed fish, upon infection or not, was investigated. The resulting differentially expressed proteins were clustered depending on their biological meaning and visualized on metabolic pathway maps, with special emphasis on immunity. We hypothesized that prolonged exposure to low hyperosmotic stressor may trigger the inflammatory response, leading to activation of innate immunity and confer protection against bacterial disease.

2. Material and method

2.1 Experimental design and statistical rationale

Investigations have been conducted following the guidelines for animal care and use in compliance with European regulation on animal welfare, protocol n°KE 12/189. Statistical rationale of sample size was based on power consideration $Z_\beta = 0,84$ with $\alpha=5\%$. One-week old striped catfish were imported in the University of Namur from the Nam Sai fish farm in Ban Sang, Thailand. Fish were maintained in recirculating aquaculture system under constant photoperiod (12L:12D) at 28°C and daily fed *ad libitum* with commercial dry pellets (Troco Supreme 16, Coppens, The Netherlands). At 3 months (initial body weight: 42±11 g), fish were transferred into two experimental circuits, each including four aerated 100-L tanks and acclimated during 10 days to these new housing conditions. **Figure 1** shows the experimental design of the experiment. The control group was maintained in freshwater (0 ppt) during the whole experiment. The experimental group was exposed to a gradual salinity increase up to 10

ppt during 20 days. In this group, salinity was daily increased (1 ppt per day) by adding natural marine salt (Instant Ocean, Belgium) mixed with tap water. On day 20, 12 fish were caught with net from each tank, anaesthetized in 150 mg L^{-1} of MS 222 and intraperitoneally injected with 0.025 ml g^{-1} fish of a bacterial solution suspended in Hank's Balanced Salt Solution (10^5 Colony Forming Unit (CFU) ml^{-1}). Mortality rate was monitored during 10 days. Moreover, during the experiment, 6 fish per tank were randomly collected with nets on day 0, day 10, day 20 and day 23 (i.e. 72 h after bacterial infection, survivors were collected) and anaesthetized. Blood was collected in the caudal vein using a sterile 1 ml heparinized syringe within 5 min after capture and then euthanized by cervical dislocation. Plasma was collected after blood centrifugation (4°C, 7 000 g, 10 min) and kept at -80°C pending analysis. Branchial left arches 1 and 2 and kidney were sampled and immediately frozen in liquid nitrogen. Oxygen level (5.7±0.5 mg L^{-1}), pH (8.4±0.24), temperature (28.2±0.1 °C) and nitrogen level ($N\text{-}NO_3^-$: 3.55±2.27 mg L^{-1}; $N\text{-}NO_2^-$: 0.019±0.005 mg L^{-1}; $N\text{-}NH_3^+$: 0.19±0.25 mg L^{-1}) were daily monitored in the outlet pipe. Measured salinities were closed to expected values (±0.15 ppt).

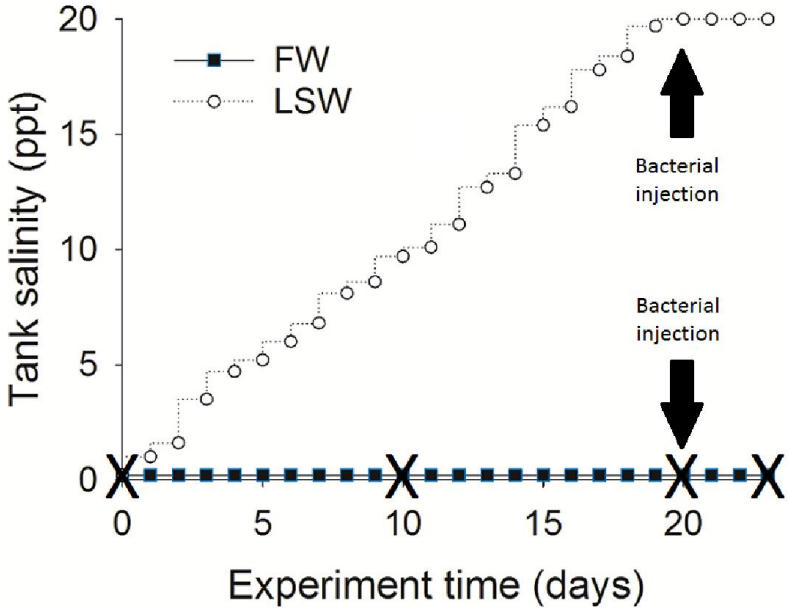


Figure 1: Experimental design of striped catfish multi-stress experiment. Fish were exposed (or not) to increasing salinity during 20 days until 10 ppt, and then to bacterial infection. Cross represents the sampling days.

2.2 Bacterial challenge

Bacterial challenges were performed according to our previous studies. Briefly, a virulent strain of *Edwardsiella ictaluri* (TNA 015, Can Tho University, Vietnam) was cultured on Brain Heart Infusion (BHI) Agar (Sigma) at 28°C. A reference bacteria solution (optical density of 0.1 at 590 nm, 10^9 CFU ml^{-1} in BHI) was numbered by serial counts of CFU on agar plates (dilution 100 to 10^{10}) as well as on black filters in fluorescent microscopy after 4'-6'-diamidino-2-phenylindole staining. Lethal dose 50% (72h), corresponding to intraperitoneal injection of 0.025 ml g^{-1} fish of a bacterial solution containing 10^6 CFU ml^{-1} was determined in a preliminary experiment. For the experiment, 10^5 CFU g^{-1} fish was injected. Infection was confirmed on the kidney by the BRLFD-CER group (Belgian Reference Laboratory of Fish Diseases-Centre d'Economie Rurale, Aye, Belgium). Kidneys of infected fish were crushed with sterile mortar and pestle and inoculated on Sheep Blood Agar. Agar plates were examined after 2 days at 26°C and demonstrated growth of apparent homogenous gram negative bacteria (after gram

stain). A 96-wells Gram Negative Biolog identification system MicroLog, version 4.0 was used for speciation. The test confirmed with 100% probability for *E. ictaluri* and 0.783 of similitude with the reference strain.

2.3 Osmoregulatory parameters

Gill filaments were homogenized in sterile Potter for 2X30 s in cold SEI buffer (Sucrose 0.25 M, EDTA 1 mM, Imidazole 50 mM, pH 7.4), containing a protease inhibitor cocktail (Sigma). Homogenates were centrifuged twice to remove main debris (10 000 g, 5 min, 4°C). Na^+K^+ ATPase activity was measured in duplicates following Mc Cormick (1993). One unit of Na^+K^+ ATPase activity represents the consumption of 1 µmole NADH min^{-1} ml^{-1}. Plasma osmolality was measured with a micro-osmometer (Type 6, Löser Messtechnik, Germany) in duplicates.

2.4 Statistical analysis

Regarding osmoregulatory parameters (i.e. blood osmolality and gill Na^+K^+ ATPase activity), heterogeneity of variances was tested by Levene test and normality was checked by Shapiro-Wilk test. Data were analysed by two-way analysis of variance ANOVA 2 (2 factors: "salinity" and "infection") followed with pairwise multiple comparisons procedures by Scheffe test ($p<0.05$) in SigmaPlot version 12. Data are represented as the mean ± SEM and tanks were used as the statistical unit (n = 4, 6 fish per tank). Regarding mortality rates, mortalities per tank were monitored each day during 10 days. Daily mortality rate in freshwater and brackish water were statistically compared using Student t-test. Data are represented as the mean± SEM (n=4, 6 fish per tank).

2.5 Quantitative label-free proteomic analysis

Proteolysis
Proteomics analysis was performed on samples of days 20 and 23. Three biological replicates were performed (n=3, experimental unit=tank) and represented a pool of 6 fish per tank. Whole kidney was grinded in 9 M Urea and sonicated. Then, 10 µg of the resulting proteins were reduced with DTT 2.8 mM (60ºC, 30 min), modified with 8.8 mM iodoacetamide in 100mM ammonium bicarbonate (dark, room temperature, 30 min) and digested in 2M Urea, 25mM ammonium bicarbonate with modified trypsin (Promega) at a 1:50 enzyme-to-substrate ratio (overnight, 37°C). An additional second digestion was performed during 4 h. Then, the tryptic peptides were desalted using C18 stage-tip (Harvards), dried and suspended in 0.1% formic acid**.**
Mass spectrometry
The resulting peptides were loaded onto a C18 trap column (0.3 x 5mm, LC-Packings) connected to a homemade capillary column (25 cm, 75 micron ID) packed with Reprosil C18-Aqua (Dr Maisch GmbH, Germany) in 0.1% formic acid in water. They were analyzed by LC-MS/MS using a Q Exactive plus mass spectrometer (Thermo) fitted with a capillary HPLC (easy nLC 1000, Thermo). The peptide mixture was resolved with a linear gradient from 5 to 28 % of 95% acetonitrile with 0.1% formic acid for 120 min, followed by a gradient of 28 to 95% during 5 min and then at 95% acetonitrile with 0.1% formic acid in water during 25 min at flow rates of 0.15 µl min^{-1}. Mass spectrometry was done in a positive mode (m/z: 350–1800) using repetitively full MS scan followed by high collision induces dissociation (HCD, at 35 normalized collision energy) of the 10 most dominant ions (>1 charges) selected from the first MS scan. A dynamic exclusion list was enabled with exclusion duration of 20s.
Data analysis

The MS raw data were analysed by the MaxQuant software (version 1.5.2.8) for peak picking and quantitation, followed by identification using the Andromeda search engine (Cox et al., 2011), searching against the Characiphysae section of the NCBI-NR database (Jan2016, 54767 proteins) with mass tolerance of 20 ppm for the precursor masses and for the fragment ions. As there are almost no protein information of *Pangasianodon hypophthalmus*, or homologous organism, the Characiphysae section that contains several fish types (mainly *Ictalurus punctatus* and *Astyanax mexicanus*), was used. Methionine oxidation was set as variable post-translational modifications and carbamidomethyl on cysteine as a static one. Minimal peptide length was set to six amino acids and a maximum of two miscleavages was allowed. Peptide and protein level false discovery rates (FDRs) were filtered to 1% using the target-decoy strategy to eliminate the identifications from the reverse database and common contaminants. The MaxQuant software was used for quantitative analysis, based on extracted ion currents (XICs) of peptides enabling quantitation from each LC/MS run for each peptide identified. As several organisms were included in the database, homolog proteins were clustered as one protein group and only proteins that were identified with at least two peptides are listed (Cox et al., 2014). Log2 transformed were performed on the intensity data in order to get a normal distribution. Missing values were replaced with 10. T-Test with Permutation-based FDR (with 250 randomization, Threshold value=0.05) was done using Perseus 1.5 between the freshwater group and the saline groups. The dataset have been deposited to ProteomeXChange Contorsium via the Pride partner repository with the dataset identifier PXD004571, username: reviewer45092@ebi.ac.uk, password: Oov046pi. Hierarchical clustering of log2 intensities using Euclidian distances and average linkage was performed in Permutmatrix, a free bioinformatics platform developed by Caraux & Pinloche (2005). The Database for Annotation, Visualization and Integrated Discovery Beta (DAVID) (version 6.8) was used to enrich functionally-related proteins categories and visualize proteins on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps (Huang et al., 2009). The limited proteomic resources and annotation of the species selected for our study hampered the analysis and interpretation of our data. First, the annotation of the Andromeda search engine was heterogeneous, such that we needed to unify the proteome annotation. Homogeneous annotation was performed by homology with the zebrafish, using GI accession numbers. Proteins GI accession numbers were submitted to DAVID interface in order to perform the enrichment analysis of the ontologies and pathway mapping (EASE score <0.1). Since the results of this analysis can vary depending on the length of the list of proteins entered in DAVID, the enrichment analysis was computed with high stringency in order to avoid contamination of KEGG pathways maps by too general proteins.

Validation

Using Speed Mill Vac Bound Homogenizer, kidney lysates were obtained by homogenizing kidney tissue for 2x30s in the following buffer (1:3): Tris-HCl 50mM, NaCl 150 mM, SDS 0.1%, Triton X-100 0.1%, apopritin 0.001 mg ml^{-1}, pH 8. Lysates were then sonicated 3x10 s at 45 kHz and 5x1s at 65 kHz on ice and centrifuged at 10 000 g for 10 min to remove main debris. Total protein abundance in the samples was measured using Pierce 660nm Protein Assay Reagent (22660, ThermoScientific). Western blot analyses were performed to validate the specificity of the antibodies following the protocol of Schmitz et al. (2016c), using anti-Cdc42 610929 (BD Biosciences), anti-vinculin V9131 (Sigma) and anti-Raf1 antibodies 610151 (BD Biosciences) 1/2000. Quantification was performed by dot blotting following Schmitz et al.(2016c). Briefly, 1 µl of proteins extract was spotted in triplicates to the prewetted PVDF membrane and allowed drying out during 30 min at room temperature, blocked in PBS-Tween containing 5% skimmed milk during 1h at room temperature and probed in the same manner than for western blotting. After immunodetection, a protein loading control was performed following the method of Welinder & Ekblad (2011) in Coomassie Brilliant Blue R-250

(Biorad). Dot quantification was done using Image J software. Data were analysed by two-way analysis of variance ANOVA 2 followed with pairwise multiple comparisons procedures by Scheffe test ($p<0.05$) in SigmaPlot version 12. Data are represented as the mean ± SEM (n = 3).

3. Results

3.1 Osmoregulatory response of catfish to chronic saline stress

The osmoregulatory capacity of striped catfish was investigated through plasma osmolality (**Figure 2 A**) and gill Na^+K^+ ATPase (**Figure 2 B**). Plasma osmolality of fish kept in freshwater averaged 265±2.7 mosm L^{-1} and significantly increased in brackish water to reach up to 292±1.8 mosm L^{-1} at 10 ppt ($p<0.001$). Infection did not induce significant change in plasma osmolality. Na^+K^+ ATPase activity did not significantly vary between fish in freshwater and brackish water on days 0, 10 and 20 and was comprised between 0.28 and 0.64 U mg^{-1} gill min^{-1}. In infected fish (day 23), Na^+K^+ ATPase significantly decreased up to 0.45±0.06 U mg^{-1} gill min^{-1} in brackish water compared to freshwater values at 0.69±0.076 U mg^{-1} gill min^{-1} ($p<001$).

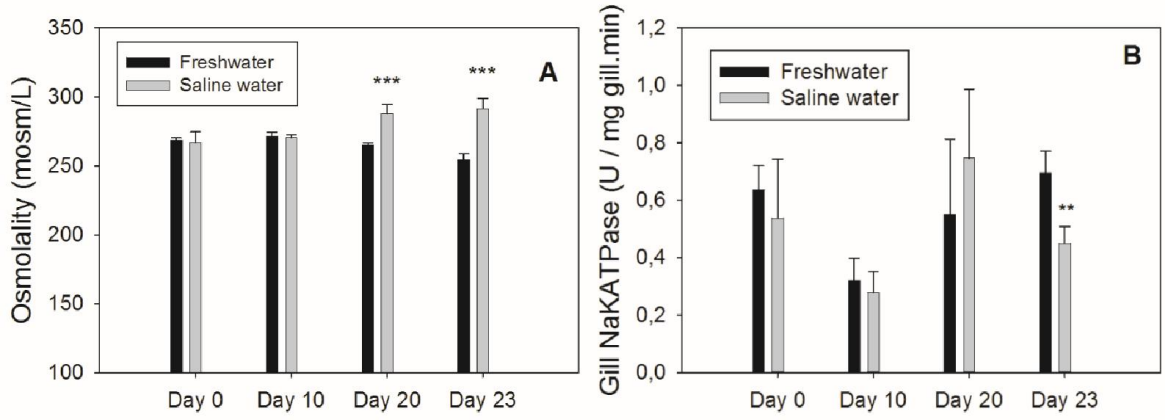


Figure 2: Osmoregulatory response of striped catfish exposed to freshwater and saline water, then infected with *Edwardsiella ictaluri*. Plasma osmolality (A) and gill Na^+K^+ ATPase activity (B) of catfish exposed to freshwater (black) and saline water gradient (0-10 ppt) (grey) during 20 days and 3 days after bacteria inoculation (day 23). The values are presented as the mean ± SD (n=4), analysed by Student's t-test. ** $p<0.01$ compared with freshwater values; *** $p<0.001$ compared with freshwater values.

3.2 Proteomic study of catfish kidney proteome in response to chronic saline stress

Hierarchical clustering of the differentially expressed proteins with salinity

Complete analysis resulted in identification of 2483 proteins, among which 400 proteins showed significant changes in abundance with elevated salinity. **Figure 3** illustrates the hierarchical clustering of the 400 differentially expressed proteins (rows) and the 4 experimental groups (freshwater and saline water, infected or not) in triplicates (columns). The experimental groups (columns) were divided into two clusters (infected or not) and the three biological triplicates in each group were clustered together. At the protein level (rows), two major clusters appeared up- or down regulated (A and B). Cluster A groups 127 differentially expressed proteins which were upregulated in infected fish raised in freshwater or saline water compared to non infected fish raised in freshwater or saline water. Cluster 2 gathers 273

differentially expressed proteins which were downregulated in infected fish raised in freshwater or saline water compared to the corresponding non infected fish.

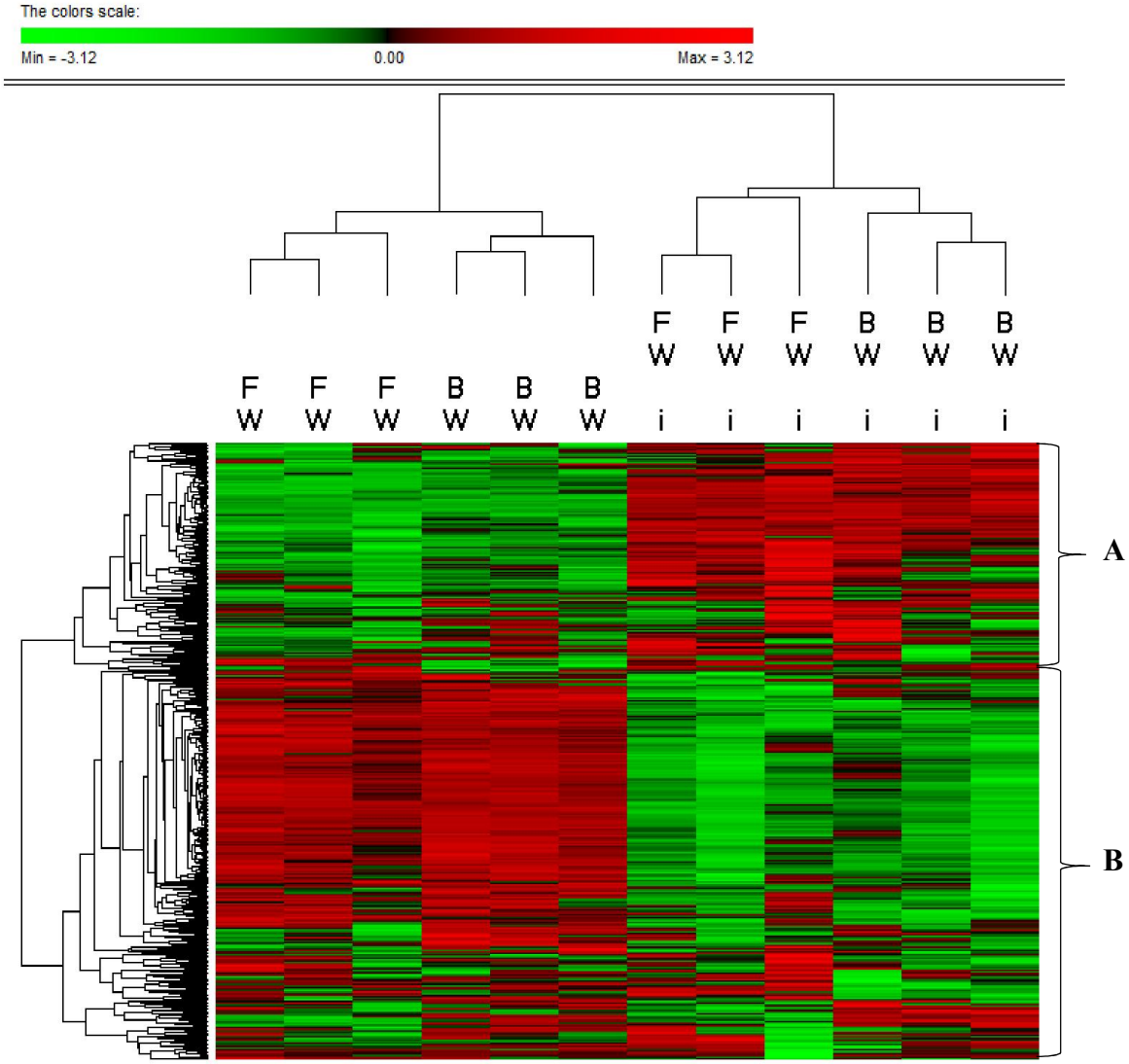


Figure 3: Hierarchical clustering of proteomic data. The hierarchical tree displays assemblies of proteins (rows) with significant changes in abundance related to salinity increase ($p<0.5$), using Pearson distances and average linkage. Log2 intensities (Z score normalized) range from low (green) to high (red) relative abundance. FW: Freshwater, not infected; BW: Saline water, not infected; FWi: Saline water, infected; BWi: Freshwater, infected.

Protein visualization on KEGG pathways maps

A pathway analysis of the whole dataset was performed to isolate the major specific functional pathways. Among the 400 differentially expressed proteins, 352 proteins were processed by DAVID interface using *Danio rerio* GI accession numbers and 156 proteins were visualized on KEGG pathway maps. **Table 1** summarizes the 25 major pathways outlined in this study, mainly involved in the energy metabolism, carbohydrate metabolism, amino acid metabolism, protein processing, immunity and detoxification.

A group of pathways related to energy production gathers enzymes involved in fatty acid beta oxidation in mitochondria and peroxisomes, glycolysis, citrate cycle, pentose phosphate pathway and ketone bodies synthesis. These enzymes generally increased with salinity from 1.2 to 2.0 fold, and decreased with infection from 1.5±0.3 fold, at the exception of fatty acid very

long chain and lactate dehydrogenase that increased with infection by respectively 1.2 and 1.5 fold. A second group of pathways gathers several enzymes implicated in amino acid metabolism, including amino acid synthesis, branched amino acid degradation and metabolism of 13 amino acids, including for example glycine, tryptophan and proline. The abundance of all enzymes listed in amino acid pathways increased with salinity up to 2.2 fold whereas it decreased with infection up to 3 fold. The next group is involved in carbohydrate metabolism and includes amino sugar, nucleotide sugar, N glycan and fructose / mannose metabolism. Enzymes listed in these pathways were generally upregulated with salinity up to 5.4 fold. Particularly, the N-acetylglucosamine kinase was upregulated up to 15.4 fold in the saline group. Nonetheless while infection rather upregulated nucleotide sugar and amino sugar metabolism, abundance of enzymes involved in fructose and mannose metabolism was depleted.

Regarding protein processing, DAVID outlined several proteins involved in COP II vesicle transport via Sec dependent pathway, folding proteins such as heat shock proteins and member of the ubiquitin ligase complex. Except for calpain and selenoprotein U (ubiquitin ligase complex) which were downregulated with infection, proteins were upregulated with salinity and infection by 1.8±0.3 fold respectively. Particularly, phospholipase A2 was strongly upregulated (6.8 fold) in saline water and infected fish.

The next group of pathways includes proteins involved in immunity and detoxification. Detoxification pathways grouped CYP 450 1A and 20 A components and enzymes involved in oxidative stress such as glutathione S transferases, glutathione peroxidase and catalase. These proteins increased by 1.7±0.5 fold with salinity but decreased by 1.8 with infection. Regarding immunity, phagosome and Salmonella infection pathways were significantly enriched. Phagosome pathway included proteins involved in the formation of phagocytic cup (e.g. coronin), phagosome acidification (V-type proton ATPases), endosomal maturation (syntaxin 7, dynein, Rab7) activation mechanisms of NAPDH oxidase (cytochrome b558, GP91 and Rac1) as well as phagolysosome transporters (Sec61 and TAP1 1) and phagocytic promotor receptors (complement receptors and integrin αMß2). The pathway is illustrated in **figure 5**. These proteins were mainly upregulated by 3 fold in salt water and during infection. Particularly, Rab 7 and syntaxin 7 increased by respectively 7.2 and 15.2 fold with salinity and 3.4 and 11.6 fold with infection and up to 7.3 and 28.9 fold when both stressors (salinity and infection) were combined. Pathway regarding Salmonella infection was significantly enriched in our study (**Figure 4**). Such as *Edwardsiella ictaluri*, Salmonella is a GRAM negative enterobacteria characterized by intracellular replication into macrophages. Therefore, Salmonella pathway in zebrafish used by DAVID may be directly compared to striped catfish infection with *Edwardsiella ictaluri*. This pathway includes members of Rho family GTPases and MAPK (mitogen-activated protein kinase) p38 signalling pathway, responsible for the proinflammatory response and the production of pro-inflammatory cytokines. In addition, proteins involved in actin cytoskeleton regulation included members of Arp2/3 complex, F actin, filamin B and dynein. Except MAPK 12 downregulated with infection by 1.3 fold, these proteins were upregulated with salinity by 2.0 fold and infection by 1.3 fold.

Several other immune proteins were highlighted such as those thought to be involved in T cell regulation and activation (i.e. plastin 2, nitrilase homolog 1, proto oncogene cRel, galectin-9, interleukin enhancer binding protein 2 and cleft lip and palate transmembrane protein 1). These proteins were upregulated by 2.0 fold with salinity and 2.5 fold with infection. Particularly, salinity upregulated by 4.6 fold the proto oncogene cRel while infection upregulated by 10.9 fold the proto oncogene cRel and by 4.0 fold the galectin-9.

Table 1: Visualization of differentially expressed proteins following low saline stress on KEGG pathway maps. This group gathered proteins of striped catfish *Pangasianodon hypophthalmus* differentially expressed ($p<0.05$) following low saline stress gradient (0-10 ppt) in infected fish or not (EASE score<0.1). Count: Proteins involved in the pathway; p-value (EASE score): to examine the significance of protein-term enrichment with a Fisher's exact test; Enrichment: measure the magnitude of enrichment; Benjamini: correct enrichment p-values to control family-wide false discovery rate.

Pathways	Count	P value	Enrichment	Benjamini
Energy production				
Glycolysis / Gluconeogenesis	20	3.3^{E}-11	6.8	1.0^{E}-9
Citrate cycle	13	7.8^{E}-8	7.1	3.6^{E}-6
Fatty acid degradation	16	6.8^{E}-8	3.7	1.4^{E}-6
Peroxisome	12	8.8^{E}-4	3.3	7.7^{E}-3
Pentose phosphate pathway	7	1.3^{E}-3	5.6	9.5^{E}-3
Synthesis and degradation of ketone bodies	7	1.8^{E}-3	5.2	1.3^{E}-2
Amino acid metabolism				
Biosynthesis of amino acid	21	7.3^{E}-11	6.1	1.83^{E}-9
Tryptophane metabolism	13	2.0^{E}-7	6.8	3.5^{E}-6
Glycine, serine, threonine metabolism	11	1.0^{E}-5	5.9	1.6^{E}-4
Histidine metabolism	8	2 $.2^{E}$-5	8.6	3.0^{E}-4
Valine, leucine, isoleucine degradation	10	1.9^{E}-4	4.8	2.3^{E}-3
Alanine, aspartate, glutamate metabolism	8	9.3^{E}-4	4.9	7.6^{E}-3
Arginine, proline metabolism	8	6.7^{E}-3	3.5	4.2^{E}-2
Lysine metabolism	7	4.3^{E}-2	2.7	1.7^{E}-1
Cysteine, methionine metabolism	5	8.7^{E}-2	2.9	2.7^{E}-1
Carbohydrate metabolism				
Amino sugar and nucleotide sugar metabolism	9	3.3^{E}-3	3.5	3.7^{E}-2
N-Glycan biosynthesis	6	4.9^{E}-2	3.0	1.9^{E}-1
Fructose and mannose metabolism	6	2.3^{E}-2	3.6	1.1^{E}-1
Protein processing				
Protein processing in endoplasmic reticulum	15	1.3 ^{E}-2	2.1	7.1^{E}-2
Protein export	6	5.6^{E}-2	4.5	2.0^{E}-1
Immunity and detoxification				
Metabolism of xenobiotics by CYP450	9	9.5^{E}-3	6.5	5.4^{E}-2
Glutathione metabolism	6	6.9^{E}-2	2.7	2.3^{E}-1
Phagosome	13	2.3^{E}-2	2.1	1.1^{E}-1
Salmonella infection	8	9.1^{E}-2	2.1	2.7^{E}-1

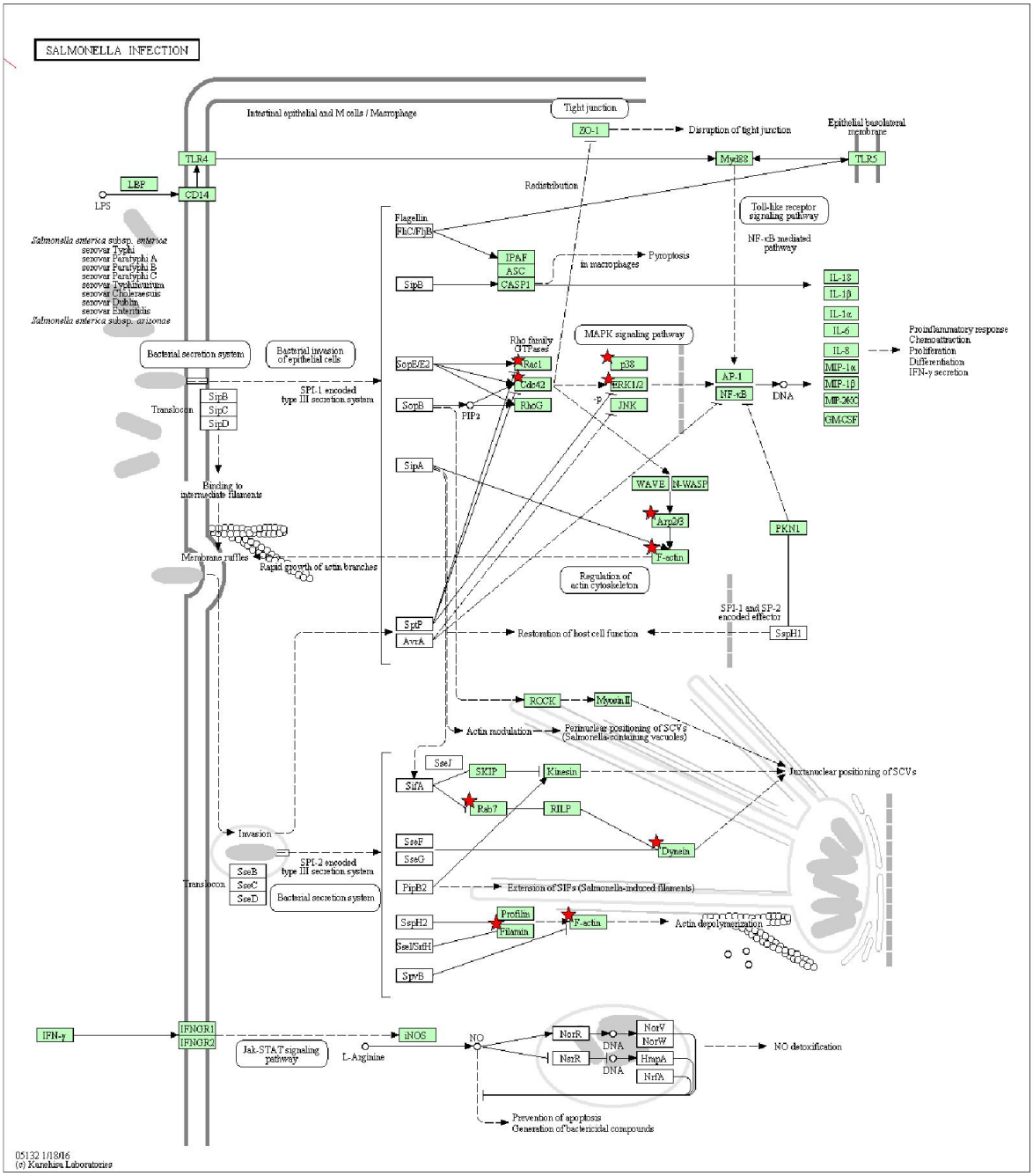


Figure 4: Differentially expressed proteins with elevated salinity visualized on *Salmonella* infection pathway map in DAVID (by homology with *Danio rerio* database). Differentially expressed proteins with elevated salinity (0-10 ppt) ($p<0.05$) are represented in red.

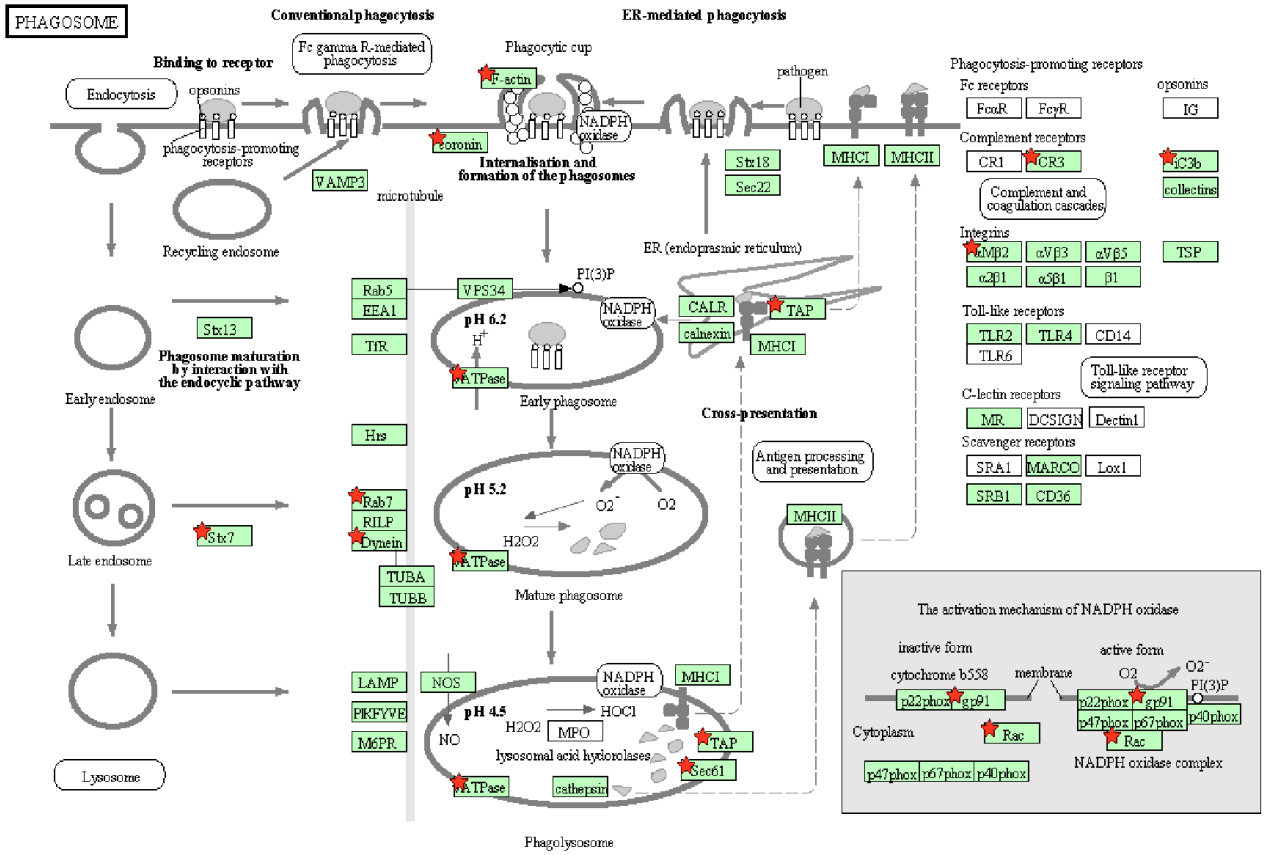


Figure 5: Differentially expressed proteins with elevated salinity visualized in phagosome maturation map in DAVID (by homology with *Danio rerio* database). Differentially expressed proteins with elevated salinity (0-10 ppt) ($p<0.05$) are represented in red.

Functional clustering of the differentially expressed proteins

A functional annotation clustering (medium stringency) was conducted on differentially expressed proteins with salinity in DAVID, using GI identifiers. Results are summarized in **Table 2**.

Nucleotide-binding proteins mainly includes ATP-binding proteins such as kinases, ion transporters, ABC transporters and ATP synthase but also G proteins and transcription factors. Particularly, RNA helicase DDX5 was enhanced up to 3 fold with salinity and up to 17 fold with infection.

Regarding protein metabolism, DAVID outlined several clusters implicated in the biosynthesis of amino acids, protein localization and transport (e.g. Small GTPases, Sec dependent pathway, cytoskeleton components), proteases (mainly proteasome subunits) and pyridoxal phosphate dependent protein modifications (aminotransferases and decarboxylases). Salinity tended to increase the abundance of these proteins. Particularly, salinity upregulated by 43.4 fold the cysteine sulfinic acid decarboxylase, involved in hypotaurine synthesis. Infection upregulated proteins involved in protein transport, localization and turnover while downregulated pyridoxal phosphate dependent transferases and proteins involved in amino acid biosynthesis.

The cluster related to ion transport includes ATP synthase subunits, calcium transporters, V-type proton ATPases, transferrin and chloride channel protein 4. Except Na/HCO3 transporter, salinity upregulated these proteins by 1.2 to 2.1 fold. Infection decreased by 1.2 fold the

abundance of ATP synthase subunits and by 2.0 fold the chloride channel protein 4 but upregulated by 3.0 fold V-type proton ATPases and by 1.2 to 1.5 fold calcium transporters. The proteins involved in the response to oxidative stress were similar to those described in the pathway analysis.
The major signalling pathway outlined by DAVID belongs to the small GTPases superfamily, Rab type (Rab 1, 2, 7, 10, 11, 21 and 25). Salinity and infection upregulated these proteins by 1.2 to 7.2 fold.
Eventually, the pathway related to cell morphology and adhesion includes actin-binding proteins involved in actin cytoskeleton organization and focal adhesions (i.e. caveolin, integrins, actin, α-actinins, vinculin, ezrin, nck-associated protein, Slingshot homolog 3, myosin and clue signal transducers such as protein RhoC, Arp2/3 complex and PAK 2). These proteins were upregulated during saline stress and infection, up to 5 fold when both stress were combined.

Table 2: Functional clustering of differentially expressed proteins following low saline stress (by homology with *Danio rerio* database)**.** This group gathers proteins of striped catfish *Pangasianodon hypophthalmus* differentially expressed ($p<0.05$) following low saline stress gradient (0-10 ppt) in infected and non-infected fish (EASE score<0.1). Count: Proteins involved in the pathway; p-value (EASE score): to examine the significance of protein-term enrichment with a Fisher's exact test; Enrichment: measure the magnitude of enrichment.

Annotation cluster	Count	P value	Enrichment
Generation of precursor metabolites and energy	18	2.7^{E}-9	5.1
Nucleic acid metabolism			
Nucleotide-binding proteins	47	2.9^{E}-6	3.7
Protein metabolism			
Biosynthesis of aminoacid	31	7.3^{E}-11	11.6
Protein localization and transport	23	2.2^{E}-2	6.6
Protease	11	3.0^{E}-2	1.7
Pyridoxal phosphate dependent transferase	7	2.0^{E}-4	2.6
Redox state			
Ion transport	5	2.5^{E}-3	2.3
Response to oxidative stress	4	2.4^{E}-2	1.7
Signalling pathway			
Small GTPase mediated signal transduction	12	4.0^{E}-2	2.7
Cell morphology and adhesion			
Actin cytoskeleton	10	1.2^{E}-5	2.5

Validation

Vinculin, Cdc42 and Raf 1 were chosen regarding their key implication in three major significant pathways outlined in proteomics (cytoskeleton/focal adhesion, MAPK kinase signalling pathway and small GTPases signalling superfamily). Dot blotting and proteomics both revealed a significant upregulation of these proteins/pathways. By dot blotting, neither salinity alone nor infection alone induced a significant change in vinculin abundance while the combination of both infection and salinity upregulated the vinculin by 1.4 fold. By proteomics, salinity alone induced upregulation by 1.3 fold, infection alone induced no significant change while the combination of both infection and salinity upregulated the vinculin by 1.3 fold. Regarding Cdc42 abundance, salinity and infection induced upregulation by 1.2 to 2.3 fold in dot blotting and by 1.3 to 1.4 fold in proteomics. Eventually, following dot blot analysis of Raf

1, salinity alone did not induce significant changes while infection induced upregulation by 1.6 fold and the combined effect of salinity and infection induced upregulation by 2.13 fold. Following proteomics results, salinity, infection and their combination induced upregulation by 1.4, 1.3 and 1.8 fold respectively.

3.3 Susceptibility to Enteric Septicaemia of Catfish

Figure 6 shows cumulative mortalities during 10 days after bacteria inoculation in the freshwater and saline water groups. Regarding daily mortality rate monitoring, no significant difference was observed between the freshwater group and the brackish water group. Mortalities begun 2 days and 3 days after bacteria inoculation in brackish water and freshwater respectively. Cumulative mortalities after 10 days reached 79±8 % in freshwater and 67±14 % in brackish water.

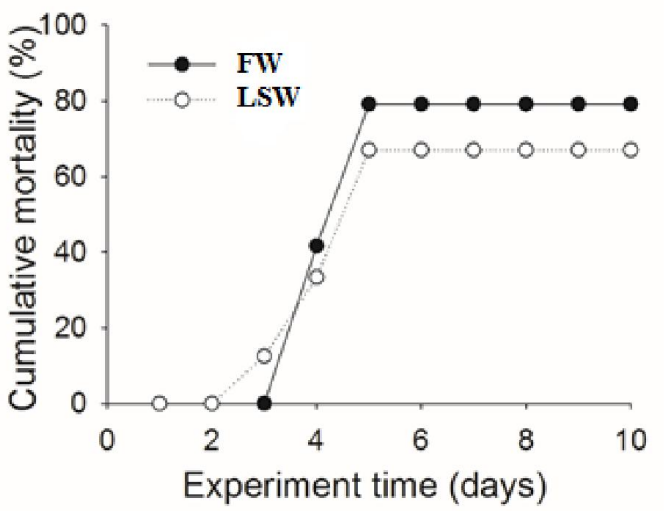


Figure 6: Cumulative mortality of striped catfish during 10 days after inoculation of *Edwardsiella ictaluri*. Mean cumulative mortality during 10 days of catfish exposed to freshwater (FW) and saline water (SW). The values are represented as the mean cumulative mortality (4 tanks, 6 fish/tank).

4. Discussion

General stress response: response to hyperosmotic stress

Plasma osmolality of fish reared in freshwater conditions were closed to those reported by other authors in catfish (Eckert et al., 2001; Phuc et al., 2014), differing by less than 10%. In this study, when salinity increased up to 10 ppt, plasma osmolality increased from 265 to 292 mosm while gill Na^+K^+ ATPase activity did not significantly vary. Under salt stress, similar responses have been described in striped catfish and channel catfish by other authors (Eckert et al., 2001; Phuc et al., 2014). However, our previous studies suggested that striped catfish are able to activate gill Na^+K^+ ATPase pumps in elevated salinities[7]. In the channel catfish, salt stress elevated plasma osmolality and increased the activity of renal glomeruli in order to produce hyperosmotic urine relative to plasma (Norton & Davis, 1976, Eckert et al., 2001).

In animal cells, increase in environmental osmolality that induces water efflux leading to cell shrinkage is counteracted by an acute stress response called “Regulatory Volume Increase”, responsible for the activation of ion transporters and retention of monovalent ions (Alfieri et al., 2007). If the saline stressor persists, inorganic ions must be replaced by organic

osmolytes in order to restore the electrochemical gradient across the cell and prevent macromolecular damages (Alfieri et al., 2007; Yancey et al., 2005). In this study, saline stressor was responsible for upregulation of ion transporters combined with a large set of proteins involved in osmolyte biosynthesis (e.g. glycerol, taurine, proline, sarcosine, betaine, spermidine, carbohydrates). The synthesis of osmotically active metabolites (i.e. aminoacids and derivatives, polyamines, carbohydrates, polyols and urea) control ion and water flux, may act as antioxidants, stabilize macromolecules and counteract perturbants in non–interchangeable ways (Yancey et al., 2005; Kumari et al., 2015). In higher salinity (20 ppt), striped catfish also upregulated its metabolism related to osmoregulation including protein synthesis, ion transporters and energy (Schmitz et al., 2016b). Uregulation of protein synthesis, energetics metabolism and ion transporters have also been described in other fish species (Martos-Sitcha et al., 2016). Regarding protein metabolism, two co- or posttranslational protein modification were especially enriched in this study and might be related to the osmotic response of the proteome: N glycosylation and pyridoxal phosphate modification. In the kidney of *Xenopus laevis*, prevention of N-glycosylation interfered with ionic transporter insertion into plasma membrane and reduced its functional expression (Paredes et al., 2006).

Following cellular stressor, eukaryotic cells are able to rearrange their cytoskeleton in order to increase cell rigidity and oppose to cell shape changes. In this study, infection and salinity upregulated various proteins related to actin network organization (e.g. WAVE complex, filamin B, Arp 2/3 complex, Rho family GTPases). In fish, responsiveness of cytoskeleton to osmotic stress has already been described by several authors (Boutet et al., 2006; Tse et al., 2014; Schmitz et al, 2016b). In eukaryotic cells, hyperosmolarity is responsible for filamentous actin polymerization and migration to periphery in order to form a thick ring under the plasma membrane to withstand with extensive cell shrinkage and ensure volume integrity (Aizawa et al., 1999; Rizoli et al., 2000; Di Ciano et al., 2006). Interestingly, number of the enriched cytoskeleton proteins in this study are connected to focal adhesion assembly (e.g. vinculin, integrin ß, α-actinins, ezrin). In rat endothelial cells, hyperosmolar exposure induced focal adhesion formation and activation of focal adhesion kinases (Quadri et al., 2003). Focal adhesions modulate various intracellular signalling pathways, especially the Arp2/3 complex and the Rho-family GTPases such as Ras and Cdc42, also significantly enriched in this study (Mitra et al., 2005). Activation of these signalling cascades by focal adhesion kinases induces filamentous actin assembly into stress fibres and protrusion forces (i.e. filopodia and lamellipodia), that function to provide the framework that supports cell motility (Mitra et al., 2005). Similarly, in a previous study, striped catfish exposed to high salinity (20 ppt) and bacterial infection upregulated actin cytoskeleton proteins and Rho family GTPases (Schmitz et al., 2016b). However, these proteins were not linked to focal adhesion assemblages. In addition, our previous results suggest a collapse of the extracellular matrix and intermediate filament network due to high salinity (Schmitz et al., 2016b). On the opposite, low salinity in this study did not induce significant changes in the abundance of extracellular matrix components whereas intermediate filaments (i.e. keratin, cytoskeleton types) rather tended to be upregulated.

Under salt stress, increase in energetic metabolism and loss of coordination between several metabolic pathways lead to the formation of excessive ROS (Kumari et al., 2015). Enhanced antioxidant defences counteract the deleterious side effects of oxidative stress in the organism. We show that low and high salinity upregulated antioxidant defences whereas infection decreased the abundance of anti-oxidizing enzymes which may lead to the increase of free ROS, a frequent observation during pathogenicity (Paiva et al., 2014, Schmitz et al., 2016b). Similarly to antioxidant defences, cytochrome P450 1A involved in detoxification process was upregulated with salinity but downregulated with infection. Upregulation of CYP450 1A have also been observed during hyperosmotic stress in seabass *Dicentrachus*

labrax,rainbow trout *Oncorhynchus mykiss* and gilthead seabream *Sparus aurata* (Ky et al., 2007; Leguen et al., 2010; Martos-Sitcha et al., 2016). These results suggested that CYP 450 1A may be implicated into salt tolerance mechanisms in fish.

Effect of salinity on immune-related pathways.

The abundance of the differentially expressed proteins in this study was first driven by the infection status and lead to two main clusters (A and B), respectively upregulated and downregulated upon infection (**Figure 4**). Then, the hierarchical clustering shows secondary clusters according to the salinity status (freshwater and brackish water).

Regarding the proteomic analysis, salinity and infection upregulated key components of the immune system involved in small GTPases and MAPK p38 signalling cascade, maturation of phagosome, actin cytoskeleton rearrangements and T cell regulation.

Phagocytosis relies on a network of endocytic vesicles to deliver cargo from new formed phagosome to lysosome for degradation (**Figure 5**). With time, phagosome is subjected to sequential fusion with endosomes and subsequent acidification during the so-called process "phagosome maturation" (Nishi & Forgac 2002; Vieira et al., 2002). This process is regulated by Toll Like Receptors through MyD88-dependent phosphorylation of MAPK p38 signalling (Blander & Medzhitov 2004). In the endocytic trafficking, Rabs family GTPases serve as master regulator in order to increase the rate of endocytic traffic (Blander et al., 2006). In this study, salinity and infection upregulated proteins involved in the formation of phagocytic cup (e.g. coronin, complement receptors, integrin α), phagosome acidification (V-type proton ATPases), phagosome maturation (syntaxin 7, dynein, Rab7), NAPDH oxidase complex (cytochrome b558, GP91 and Rac1), phagolysosome transporters (Sec61 and TAP1 1) and phagosome regulation (small GTPases, especially Rab superfamily and MAPK38 signalling pathway). In particular, Rab7 and syntaxin 7 were highly upregulated with salinity and infection up to 7 and 29 fold respectively. On one hand, Rab7 is known to mediate the redistribution of late phagosome from cell periphery to the juxtanuclear region together with microtubule-based motor dynein (Jordens et al., 2001). On the other hand, syntaxin 7 specifically induces the fusion between late endosome and lysosome (Mullock et al., 2000). Enhancement of the phagocytic process, especially the final step including phagolysosome formation, may be beneficial for the organism as intracellular bacteria such as *Salmonella* sp and *Edwarsiella* sp are thought to interfere with the phagosome maturation and inhibit the fusion between bacteria-containing phagosomes with lysosomes (Steele-Mortimer 2008). Nonetheless, polymerization of F-actin, especially in cell periphery, may favour the formation of vacuole-associated actin polymerization. Indeed, some intracellular bacteria such as *Salmonella* are known to manipulate the host cytoskeleton machinery in order to build a F-actin network in the vicinity of bacteria-containing phagosome and so generate a specialized niche for bacteria replication (Méresse et al., 2001).

Small GTPases and MAPK p38 cascade mediate various cellular responses including apoptosis, gene transcription, cytoskeletal organization, immune response (e.g. inflammatory response and cytokine storm) and growth in response to diverse extracellular signals (Cowan et al., 2003). Phosphorylation / activation of the MAPK p38 signalling pathway and upregulation of small GTPases in response to hyperosmotic stresses and infection have been described in various types of cells in many organisms including fish (Kultz & Avila 2001; Aggeli et al., 2002; Tse et al., 2014). Moreover, in brown trout *Salmo trutta* and Mozambique tilapia *Oreochromis mossambicus*, phagocytic activity of renal leukocytes increased after seawater transfer (Marc et al., 1995; Jiang et al., 2008). Nonetheless, the phagocytic pathway was not significantly impacted at higher salinities (20 ppt) in striped catfish (Schmitz et al., 2016b). On the contrary, abundance of opsonins (complement proteins) decreased in saline water compared

to freshwater level. Therefore, it seems that low saline stressor may be beneficial for the organism though enhancement of phagosome maturation if it does not lead to excessive inflammatory response. However, increase in actin cytoskeleton meshwork to the vicinity of phagosome formation and maturation might impair juxtanuclear positioning of bacteria-containing vacuoles and impair their accessibility to the host immune system.

A key immunological consequence of phagosome maturation is the presentation of foreign antigens by MHC class II, which lead to the activation of antigen-specific CD4+ T cell (Krogsgaard et al., 2005). In this study, several proteins thought to be involved in T cell activation such as plastin 2, proto oncogene cRel, galectin-9, interleukin enhancer binding protein 2 and cleft lip and palate transmembrane protein 1 were upregulated with salinity and infection. Particularly, salinity upregulated 4.6 fold the proto oncogene cRel, a Rel NF-kB family transcription factor which play a crucial function in lymphoid cells development and interleukin production via CD28-mediated signalling (Huang et al., 2001). The interaction between stress and the T cell response has been mostly studied in higher vertebrates while less information is available in fish. In higher salinity (20 ppt), proteins involved in T cell activation and regulation were not significantly differentially expressed compared to freshwater conditions (Schmitz et al., 2016b). In fish and mammals, administration of stress hormones enhanced leukocytes proliferation, trafficking and cytokine expression in organs (Maule & Schreck 1990; Dhabhar et al., 2008). In mammals, elevated plasma osmolarities (i.e. by hypertonic saline treatment or KCl injection) increased T lymphocyte proliferation and function via MAPK p38 stimulation and ATP release (Junger et al., 1994; Woerhle et al., 2010). In addition, López-Rodríguez et al.(2005) show that NFAT5 protein, which links the Rel NF-kB and NFAT families, regulates the production of specific cytokines in T cells during osmotic stress. Therefore, it might be possible that similarly to mammals, elevated plasma osmolality enhances T cells proliferation and/or activity at least partly via the cRel / NF-kB family transcription factor.

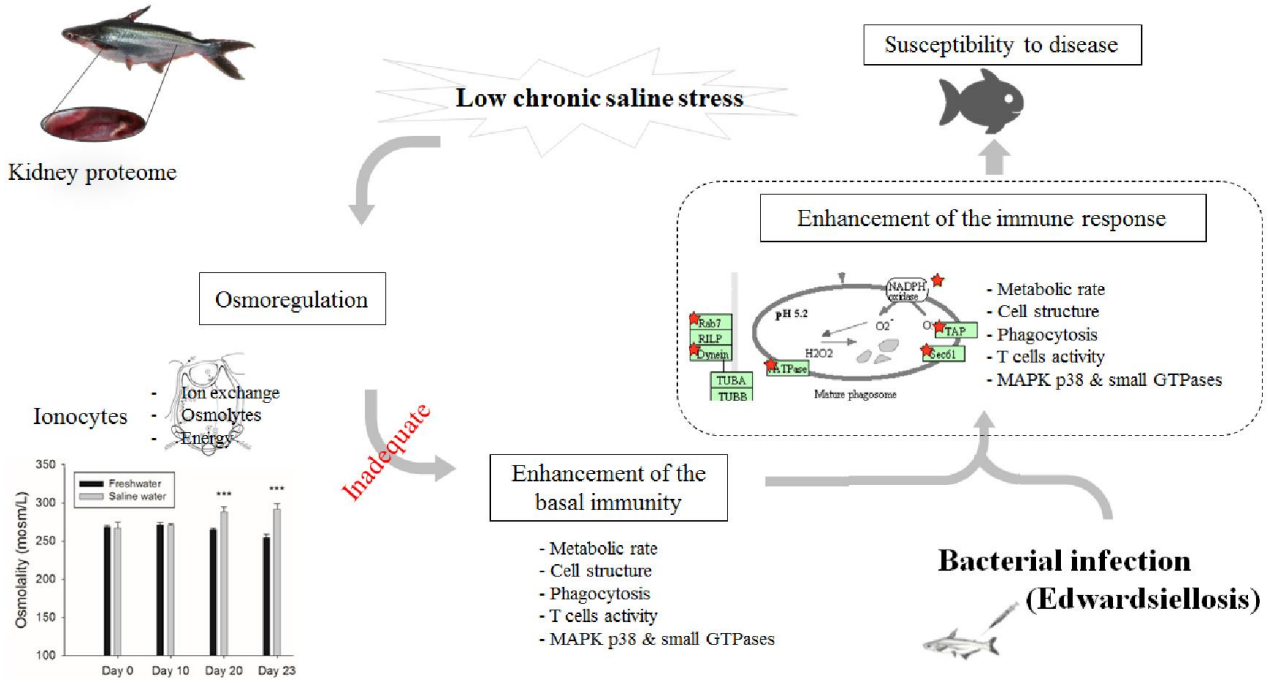
Regarding susceptibility to ESC, cumulative mortality after infection was not significantly affected by the salinity (**Figure 6**). On the contrary, higher salinities up to 20 ppt induced excessive inflammation leading to tissue destruction and increased susceptibility to ESC (Schmitz et al., 2016b).

The osmolarity of most physiological fluids is regulated within a narrow window of tolerance regarding osmotic fluctuations. Fish and mammalian tissues have developed sensory and signalling systems which monitor the tissue barrier integrity and serves as pro-inflammatory stimuli during tissue damage (Enyedi et al., 2013). Whether hyperosmotic stressors might be perceived as a danger signal by disrupting constitutive local osmotic gradients is unknown. However, it is likely that increased internal body pressure unbalances the osmotic homeostasis of the organism, leading to the activation of osmotic surveillance signalling pathway, thereby trigerring inflammatory responses and immune cells activation. Modulation of the immune system in response to changes in media salinity might also be indirectly induced by the hormonal control of the osmotic homeostasis or by a shift in the fish microbiota. Further studies are needed to understand the link between environmental salinity, osmoregulation and immunity.

The use of heterologous databases in quantitative proteomics

Proteomics studies in non-model organisms are hampered by the lack of fully annotated, detailed and high quality proteome and thereby limit the value of a proteomic approach for protein identification and quantitation (Arnold &Frohlich, 2011). Protein identification is based on cross-species matching and thus success is reliant on the rate of divergences of protein sequences and the taxonomic proximity from higher quality proteome. Therefore, cross-species

comparisons may weaken the confidence of the identification and decrease the number of proteins considered in our study. This explains for instance the relatively poor sequence coverage compared to similar analysis in model species. In addition, the requirements for precursor ion mass matching and product ion alignments induce that only conserved regions of the protein can elicit match (Bayram et al., 2016). In consequences, rapidly evolved proteins may be less abundant than slowly evolving proteins which matched better with phylogenetic neighbours (Bayram et al., 2016). In addition, the functional classification was clarified by an analysis of the proteome using KEGG metabolic pathway maps, using the DAVID resources. As accurate pathway maps were only available for the zebrafish and the annotation of the Andromeda search engine was heterogeneous, we needed to unify the proteome annotation by homology with the zebrafish. Due to the lack of correspondences, this step constituted a significant unspecific filter and the number of proteins considered in our analysis was limited.



5. Conclusions

This study provided novel insights into the molecular mechanisms that regulate the hyperosmotic response in kidney of stenohaline fish. In addition, a multi-stress approach has been applied in order to study the cross responses between osmoregulatory and immune pathways.

The differentially expressed proteins were mainly driven by the infectious status while the low saline stress in this study induced similar responses in non-infected and infected fish. In this study, low salinity upregulated various proteins involved in common metabolic pathways including mainly the energetics metabolism, protein metabolism and transport, actin cytoskeleton and detoxification. Regarding immunity, salinity upregulated proteins involved in Rab family and MAPK p38 pathway, phagocytosis and T cells regulation in non-infected and infected fish.

To conclude, low salinity may have a protective role by moderately enhancing the immune system and the immune response to disease. Comparatively, our previous results showed that high salinity (20 ppt) may induce excessive inflammation that may end up to tissular destruction, sepsis and eventually increased the susceptibility to bacterial disease.

Acknowledgements: We are grateful to FRIA (Fonds de la Recherche dans l'Industrie et l'Agriculture, Wallonia-Brussels Federation) for providing a PhD grant to Mélodie Schmitz. Authors also thank the BELSPO funded project IAP Aquastress (P7/31).

References

Aizawa, H., Katadae, M., Maruya, M., Sameshima, M., Murakami-Murofushi, K., Yahara, I. Hyperosmotic stress-induced reorganization of actin bundles in Dictyostelium cells over-expressing cofilin. *Genes Cells,* 1999, *4,* 311–324.

Aggeli, I., Gaitanaki, C., Lazou, A., Beis, I. Hyperosmotic and thermal stresses activate p38-MAPK in the perfused amphibian heart. *J. Exp. Biol.,* 2002, *205,* 443-454.

Alfieri, R., Petronini, P.G. Hyperosmotic stress response: comparison with other cellular stresses. *Pfülgers Archiv.,* 2007, *454*, 173-185.

Arnold, G., Frohlich, T. Dynamic proteome signatures in gametes, embryos and their maternal environment. *Reprod. Fertil. Dev*. 2011, *23,* 81–93.

Bayram, H. L., Claydon, A. J., Brownridge, P. J., Hurst, J. L., Mileham, A., Stockley, P., Beynon, R., Hammond, D.E. Cross-species proteomics in analysis of mammalian sperm proteins. *J. Proteomics* 2016, *135*, 38-50.

Blander, J., Medzhitov, R. On regulation of phagosome maturation and antigen presentation. *Nat. Immunol.,* 2006, *7,* 1029-1035.

Blander, J., Medzhitov, R. Regulation of phagosome maturation by signals from Toll-like receptors. *Science* 2004, *304*, 1014-1018.

Boutet, I., Long Ky, C.L., Bonhomme, F. A transcriptomic approach of salinity response in the euryhaline teleost, *Dicentrachus labrax*. *Gene,* 2006, *379*, 40-50.

Caraux, G., Pinloche, S. Permutmatrix, a graphical environment to arrange gene expression profiles in optimal linear order. *Bioinformatics,* 2005, *21*, 1280-1281.

Chen, J., Wu, H., Shi, Y., Li, C., Li, M. The effects of environmental salinity on trunk kidney proteome of juvenile ayu (*Plecoglossus altivelis*). *Comp. Biochem. Phys. D,* 2009, *4*, 263-267.

Chou, H.Y., Peng, T.Y., Chang, S.J., Hsu, Y.L., Wu, J.L. Effect of heavy metal stressors and salinity shock on the susceptibility of grouper (*Epinephelus* sp.) to infectious pancreatic necrosis virus. *Virus Res.,* 1999, *63,* 121-129.

Cox, J., Hein, M.Y., Luber, C.A., Paron, I., Nagaraj, N., Mann, M. Accurate Proteome-wide Label-free Quantification by Delayed Normalization and Maximal Peptide Ratio Extraction, Termed MaxLFQ. *Mol. Cell. Proteomics,* 2014, *13*, 2513-2526.

Cox, J., Neuhauser, N., Michalski, A., Scheltema, R.A., Olsen, J.V., Mann, M. Andromeda – a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res*., 2011, *10*, 1794-1805.

Cuesta, A., Laiz-Carrion, R., Martin del Rio, M., Meseguer, J., Mancera, J., Esteban, M. The salinity influences humoral immune parameters of gilthead seabream (*Sparus aurata*). *Fish Shellfish Immun.,* 2005, *18*, 255-261.

Dey, H., Liu Z.R. Phosphorylation of P68 RNA Helicase by P38 MAP kinase contributes to colon cancer cells apoptosis induced by oxaliplatin. *BMC,* 2012, *13*, 1-8.

Dhabhar, F.S. Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection versus immunopathology. *Allergy Asthma Clin. Immunol*., 2008, *4,* 2–11

Di Ciano-Oliveira, C., Thirone, A.C.P., Szászi, K., Kapus, A. Osmotic stress and the cytoskeleton: the R(h)ole of Rho GTPases. *Acta Physiol*., 2006, *187,* 257–272.

Eckert, S.M., Yada, T., Shepherd, B.S., Stetson, M., Hirano, T., Grau, E.G. Hormonal control of osmoregulation in the channel catfish Ictalurus punctatus. *Gen. Comp. Endocr*., 2001, *122*, 270–286.

Enyedi, B., Kala, S., Nikolich-Zugich, T., Niethammer, P. Tissue damage detection by osmotic surveillance. *Nat. Cell. Biol.,* 2013, *15*, 1123-1132.

FAO – Food and Agriculture Organization of the United Nation. The state of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations. Rome, 2014, pp. 243.

Flowers, T.J., Colmer, T.D. Plant salt tolerance: adaptations in halophytes. *Ann. Bot.,* 2015, *115*, 327–331.

Huang, D., Chen, Y., Ruetsche, M., Phelps, C., Ghosh, G. X-ray crystal structure of proto-oncogene c-Rel bound to the CD28 response element of IL2. *Structure,* 2001, *9*, 669-678.

Huang, D.W.H., Sherman, B.T., Lempicki, R.A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc*., 2009*, 4*, 44-57.

Jiang, I-F., Khumar, V.B., Lee, D.N., Weng, C.F. Acute osmotic stress affects tilapia (*O. mossambicus*) innate immune responses. *Fish Shellfish Immun*, 2008, *25,* 841-846.

Jordens, I., Borja, M., Marsman, M., Dusseljee, S., Janssen, L., Calafat, J., Janssen, H., Neefjes, J. The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein dynactin motors. *Curr. Biol*., 2001, *11*, 1680-1685.

Junger, W.G., Liu, F.C., Loomis, W.H. Hypertonic saline enhances cellular immune function. *Circ. Shock* 1994, *42*, 190–196.

Kalujnaia, S., McWilliam, S., Zaguinaiko, V.A., Feilen, A.L., Nicholson, J., Hazon, N., Cutler, C.P., Cramb, G. Transcriptomic approach to the study of osmoregulation in the European eel Anguilla anguilla. *Physiol. Genomics,* 2007, *31,* 385-401.

Kang, J., Frank, J., Kang, C., Kajiura, H., Vikram, M., Ueda, A., Kim, S., Bahk, J., Triplett, B., Fujiyama, K., Lee, S., von Schaewen, A., Koiwa, H. Salt tolerance of *Arabidopsis thaliana* requires maturation of N glycosylated proteins in the Golgi apparatus. *PNAS,* 2008, *105*, 5933-5938.

Krogsgaard, M., Li, Q.J., Sumen, C., Huppa, J., Huse, M., Davis, M. Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity. *Nature,* 2005, *434*, 238–243.

Kültz, D., Li, J., Gardell, A., Sacchi, R. Quantitative molecular phenotyping of gill remodelling of gill in a cichlid fish responding to salinity stress. *Mol. Cell. Proteomics,* 2013*, 12,* 3962–3975.

Kultz, D., Avila, K. Mitogen-activated protein kinases are in vivo transducers of osmosensory signals in fish gill cells. *Comp. Biochem.Physiol. B,* 2001, *129*, 821-829.

Kumari, A, Das, P., Parida, A.K., Agarwal, P.K. Proteomics, metabolomics and ionomics perspectives of salinity tolerance in halophytes. *Front. Plant. Sci*. 2015, *6,* 1-20.

Ky, C.L., De Lorgeril, J., Hirtz, C., Sommerer, N., Rossignol, M., Bonhomme, F. The effect of environmental salinity on the proteome of the sea bass (*Dicentrachus labrax* L.). *Anim. Genet.,* 2007, *38*, 601-608.

Leguen, I., Odjo, N., Le Bras, Y., Luthringer, B., Baron, D., Monod, G., Prunet, P. Effect of seawater transfer on CYP1A gene expression in rainbow trout gills. *Comp. Biochem. Physiol.* A, 2010, *156,* 211-217.

Marc, A., Quentel, C., Severe, A., Le Bail, P., Boeuf, G. Changes in endocrinal and non-specific immunological parameters during seawater exposure of brown trout. *J. Fish Biol., 1995, 46,* 1065-1081.

Martos-Sitcha, J.A., Mancera, J.M., Calduch-Giner, J.A. , Yufera, M. , Martinez-Rodriguez, G. , Pérez-Sanchez, J. Unraveling the tissue-specific gene signatures of gilthead seabream (*Sparus aurata* L.) after Hyper- and Hypo-osmotic challenges. *PLoS ONE*, 2016, *2*, e0148113.

Maule, A. G., Schreck, C. B. Changes in numbers of leukocytes in immune organs of juvenile coho salmon after acute stress or cortisol treatment. *J. Aquat. Anim. Health,* 1990, *2*, 298–304.

Mc Cormick, S.D. Methods for nonlethal gill biopsy and measurement of NaK-ATPase activity. *Can. J. Fish. Aquatic Sci.* 1993, *50*, 656-658.

Méresse, S., Unsworth, K., Habermann, A., Griffiths, G., Fang, F., Lorenzo, M., Waterman, S., Gorven, J., Holden, D. Remodelling of the actin cytoskeleton is essential for replication of intravacuolar Salmonella. *Cell. Microbiol.*, 2001, *3,* 567-577.

Mitra, S., Hanson, D., Schlaepfer, D. Focal adhesion kinase integrates signals to promote cell migration. *Nat.Cell Biol*., 2005, *6*, 56-68.

Steele-Mortimer, O. The Salmonella containing vacuole – Moving with the times. *Curr. Opin. Microbiol*., 2008, *11,* 38-45.

Mullock, B.M., Smith, C.W., Ihrke, G., Bright, N.A., Lindsay, M., Parkinson, E.J., Brooks, D.A., Parton, R.G., James, D.E., Luzio, J.P., Piper, R.C. Syntaxin 7 is localized to late endosomal compartments, associates with Vamp 8, and is required for late endosome-lysosome fusion. *Mol. Biol. Cell*., 2000, *11*, 3137-3159.

Munnik, T., Meijer, H.J. Osmotic stress activates distinct lipid and MAPK signalling pathways in plants. *FEBS Lett*., 2001, *498*, 172-178.

Nishi, T., Forgac, M. The vacuolar (H+)-ATPases—nature's most versatile proton pumps. *Nat. Rev. Mol. Cell. Biol.,* 2002, *3*, 94-103.

Nguyen, A.L., Dang, V.H. , Bosma, R.H. , Verreth, J.A. , Leemans, R. , De Silva, SS. Simulated impacts of climate change on current farming locations of striped catfish (*Pangasianodon hypophthalmus*, Sauvage) in the Mekong Delta, Vietnam. *Ambio,* 2014, *43*, 1509-1568.

Paiva, C., Bozza, M. Are reactive oxygen species always detrimental to pathogens? *Antioxid. Redox Signal.* 2014*, 20,* 1000-1037.

Paredes, A., Plata, C., Rivera, M., Moreno, E., Vazquez, N., Munoz-Clares, R., Hebert, S., Gamba, G. Activity of the renal Na K 2Cl cotransporter is reduced by mutagenesis of N glycosylation sites: role for protein surface charge in Cl- transport. *APS Journals,* 2006, *290*, 1094-1102.

Phuc, T.H.N., Huong, D.T.T., Mather, P.B., Hurwood, D.A. Experimental assessment of the effects of sublethal salinities on growth performance and stress in cultured tra catfish (*Pangasianodon hypophthalmus*). *Fish Physiol. Biochem*., 2014, *40,* 1839-1848.

Quadri, S.K., Bhattacharjee, M., Parthasarathi, K., Tanita, T., Bhattacharya J. Endothelial barrier strengthening by activation of focal adhesion kinase. *J. Biol. Biochem.,* 2003, *278,* 13342-13349.

Rizoli, S.B., Rotstein, O.D., Parodo, J., Phillips, M.J., Kapus, A. Hypertonic inhibition of exocytosis in neutrophils: central role for osmotic actin skeleton remodeling. *Am. J. Physiol. Cell. Physiol.,* 2000*, 279, C619–C633.*

López-Rodríguez, C., Aramburu, J., Jin, L., Rakeman, A.S., Michino, M., Rao, A. Bridging the NFAT and NF-κB Families: NFAT5 Dimerization Regulates Cytokine Gene Transcription in Response to Osmotic Stress. *Immunity,* 2005, *15*, 47-58.

Schmitz, M., Baekelandt, S., Tran Thi, L.K., Mandiki, S., Douxfils J., Thinh, N., Huong, D.T.T., Kestemont, P. Osmoregulatory and immunological status of the pond-raised striped catfish (*Pangasianodon hypophthalmus* S.) as affected by seasonal runoff and salinity changes in the Mekong Delta, Vietnam. *Fish Physiol Biochem,* 2016a doi:10.1007/s10695-016-0266-7.

Schmitz, M., Mandiki, S., Douxfils, J., Ziv, T., Admon, A., Kestemont, P. Synergic stress in striped catfish exposed to chronic salinity and bacterial infection: effects on kidney protein expression profile. *J. Proteomics,* 2016b, *142,* 91-101.

Schmitz, M., Douxfils, J., Mandiki, S., Morana, C., Baekelandt, S., Kestemont, P. Chronic hyperosmotic stress interferes with immune homeostasis in striped catfish (*Pangasianodon hypophthalmus*, S.) and leads to excessive inflammatory response during bacterial infection. *Fish Shellfish Immunol.,* 2016c, *55,*550-558.

Tse, W., Sun, J., Zhang, H., Lai, K., Gu, J., Qiu, J., Wong, C. iTRAQ-based quantitative proteomic analysis reveals acute hypo-osmotic responsive proteins in the gills of Japanese eel (*Anguilla japonica*). *J. Proteomics*, 2014, *105,* 133-143.

Vieira, O.V., Boltelho, R.J., Grinstein, S. Phagosome maturation: aging gracefully. *Biochem. J.,* 2002, *306,* 689-704.

Von Schaewen, A., Frank, J., Koiwa, H. Roles of complex N glycans in plant stress tolerance. *Plant Signal. Behav.,* 2008, *3,* 871-873.

Woerhle, T., Yip, L., Manohar, M., Sumi, Y., Yao, Y., Chen, Y., Junger, W. Hypertonic stress regulates T cell function via pannexin-1 hemichannels and P2X receptors. *J. Leukoc. Biol*., 2010, *88,* 1181-1189.

Yada, T., Azuma, T., Takaji,,Y. Stimulation of non-specific immune functions in seawater-acclimated rainbow trout *Oncorhynchus mykiss*, with reference to the role of growth hormone. *Comp. Biochem. Phys*. *B,* 2001, *129*, 695-701.

Yancey, P. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J. Exp. Biol*., 2005*, 208*, 2819-2830.

Chapter 5

Synergic stress in striped catfish (*Pangasianodon hypophthalmus*, S.) exposed to chronic salinity and bacterial infection: effects on kidney protein expression profile.

Schmitz, M., Mandiki, S.N.M., Douxfils, J., Ziv, T., Admon, A., Kestemont, P. Synergic stress in striped catfish (*Pangasianodon hypophthalmus*, S.) exposed to chronic salinity and bacterial infection: effects on kidney protein expression profile. *J. Proteomics,* 2016, *142*, 91-101.

Abstract

Hyperosmotic stress has often been investigated from osmoregulation perspectives while the effects of such stress on the immune capacity remain largely unexplored. In this study, striped catfish were submitted to a gradual hyperosmotic stress (up to 20 ppt), followed by an infection with a virulent strain of an intracellular bacteria, *Edwardsiella ictaluri*. Physiological parameters (plasma osmolality, gill NaK ATPase) have been investigated. Moreover, a label free quantitative proteomics workflow was used to study how salinity affects the proteome of kidney of no infected and infected fish. The flow consisted in initial global profiling of relative peptide abundances (by LC/MS, peak area quantification based on extracted ion currents) followed by identification (by MS/MS). Differentially expressed proteins were analyzed in DAVID interface to isolate functional pathways involved in the response to bacterial infection and which are influenced by environmental salinity changes. In response to hyperosmotic stress, plasma osmolality increased while gill Na^+K^+ ATPase slightly increased. In kidney proteome, 2024 proteins were identified, among which 496 proteins were differentially expressed. Hierarchical clustering analysis leads to isolation of 4 main clusters. Several pathways and functional categories, mostly related to cell metabolism, response to stress, cell structure, immunity and ion homeostasis were highlighted and discussed.

1. Introduction

Within less than 20 years, the striped catfish (*Pangasianodon hypophthalmus*, Sauvage) has become one of the top 10 fish species cultivated worldwide. Vietnam, and more specifically the Mekong delta area, is by far the world's largest producer of striped catfish with 1.2 million of tons in 2014, representing 1.77 billion US$ in the international food market (FAO, 2014; VASEP, 2015). Since 2004, several studies have pointed out the rate of sea-level rise in the Mekong River and the great concern regarding the impact of climate changes on the agricultural development of the region (Wassman et al., 2004; Nguyen et al., 2008; Eastham et al., 2008; Nguyen et al., 2014). In the South Mekong Basin, long-term projections of climate change for 2030 predicted an increase in mean temperature (+0.79°C) and precipitation (+51-100mm/year) compared to historical mean data (1951-2000) (Nguyen et al., 2014). Saltwater intrusion in the Delta up to 40 km far from the river mouth induces elevated salinity in catfish ponds and interacts with catfish immune status, as indicated by some haematological and immunological biomarkers (Nguyen et al., 2008).

Acclimation mechanisms from hyposmotic to hyperosmotic environment (relative to fish plasma) have been investigated mainly in terms of changes in the osmoregulatory system while the effects of an osmotic stress on the immune capacity remain largely unexplored. In fish, it has been demonstrated that hyperosmotic stress may activate several key factors of the basal immune system such as the activity of lysozyme, alternative complement pathway, peroxidase and respiratory burst (Yada et al., 2001; Cuesta et al., 2005; Jiang et al., 2008) but may also in turn induce higher susceptibility to infectious diseases (Chou et al., 1999).

Proteomics is one of the main approaches to gain global insight into molecular responses to environmental stressors. Indeed, environmental stressors have been shown to induce modification of the proteome expression in different tissues of aquatic animals (Gillardin et al., 2009; Silvestre et al., 2010; Kültz et al., 2013; Ralston-Hooper et al., 2013). In gills of Mozambique tilapia *Oreochromis mossambicus*, Fiol et al.(2006) outlined activation of six major processes including stress response, osmolyte accumulation, energy metabolism, lipid metabolism, cytoskeleton and protein and mRNA stability after an acute hyperosmotic stress. Comparatively, black tilapia exposed to gradual rise in environmental salinity increased the number and size of gill chloride cells, elevated activities of specific ion transporters and a higher production of signalling chaperones (Kültz et al., 2013). In the trunk kidney, Chen et al.(2009) identified 10 differentially expressed proteins involved in energy metabolism, cytoskeleton, iron trafficking, protein metabolism and vitamin A trafficking three weeks after transfer of juvenile ayu *Plecoglossus altivelis* from freshwater to saline water.

In proteomics, the development of nano-flow liquid chromatography associated with high resolution mass spectrometer has enabled large-scale relative and absolute quantitation of proteins. In this study, the workflow consists on spectrometry-based label free quantitative (LFQ) proteomics. The suitability of LFQ approach for quantitative proteomics has already been debated in a large panel of independent studies (Cutillas et al., 2007; Wang et al., 2012; Kültz et al., 2013). Quantitation of ion current intensity is used for relative quantitation of protein abundances in kidney tissue of fish raised in chronic hyperosmotic stress compared to control fish raised in freshwater. Then, the chronic saline stress will be combined with bacterial infection. Kidney is a suitable organ for proteomics studies as it is a key organ at the crossroads of several major biological systems such as osmoregulation and immunity.

In this study, striped catfish *Pangasianodon hypophthalmus* were submitted to gradual hyperosmotic stress during 20 days up to 20 ppt. Hyperosmotic stress was followed by an infection with a highly virulent strain of an intracellular gram negative bacteria, *Edwardsiella ictaluri*, responsible for Enteric Septicemia of Catfish (ESC) (Hawke et al., 1981). ESC is considered as the most economically important cause of mortality in catfish industry, especially in fingerling production (USDA, 2011). We hypothesized that prolonged exposure to high hyperosmotic stress may induce chronic sterile inflammation and disruption of immune homeostasis that may lead to excessive inflammatory response and serious ESC disease.

2. Material and method

2.1 Experimental design and statistical rationale.

Investigations have been conducted according to the guidelines for animal use and care in compliance with Belgian and European regulation on animal welfare, ethical protocol n°KE 12/189. Rationale calculations of sample size have been based on power consideration (Z_β = 0,84, α=5%). One week old striped catfish were provided by the Nam Sai catfish farm (Ban Sang, Thailand). Juveniles were maintained in experimental facilities in the University of Namur (Belgium) at 28°C under constant photoperiod (12L:12D) in recirculating aquaculture system . Fish were fed daily *ad libitum* with commercial dry pellets (Troco Supreme, Coppens,

The Netherlands). At 3 months, fish (40-50 g) were divided into two experimental groups including each four aerated 100 L tanks (30 fish/tank). Fish were acclimated to the new housing conditions for 10 days before the start of the experiment. The control fish were maintained in freshwater (0 ppt) during the entire experiment. The second group of fish was exposed to a gradual salinity increase of 1 ppt per day during 20 days; therefore reaching a salinity level of 20 ppt by the end of the exposure period (**Figure 1**). Salinity was daily increased by adding natural marine salt mixed with tap water until day 20 and remained stable during the bacterial challenge. On day 20, fish (12 fish/tank, 4 tanks/group) were anaesthetized in MS 222 (150 mg L^{-1}) and intraperitoneally injected with 0.025 ml g^{-1} fish of a bacterial solution (10^6 bacteria ml^{-1}) suspended in Hank's Balanced Salt Solution (HBSS). Fish (6 fish/tank, 4 tanks/group) were sampled at the following times: day 0, day 10, day 20 and day 23 (i.e. 72 h after bacterial infection). Fish were randomly collected from tanks with nets and anaesthetized in MS 222 (150 mg L^{-1}). Blood was collected by caudal vein puncture using a sterile 1 ml heparinized syringe within 5 min after capture from the tank, then fish were euthanized by cervical dislocation. Blood was kept on ice until plasma was separated by centrifugation (4°C, 10 000 g, 10 min) and frozen at -80°C pending analysis. The whole kidney was rapidly collected by gentle scratching with tweezers along the vertebral column and immediately frozen in liquid nitrogen. Gill filaments from left arches 1-2 were taken out and immediately frozen in liquid nitrogen. Physicochemical data were measured daily with multiparameter probe (WTW, Multi 350i) in the outlet pipe: O_2:5.7±0.5 mg L^{-1}; pH: 8.4±0.24; temperature: 28.2±0.1 °C, N-NO_3^-: 3.55±2.27 mg L^{-1}; N-NO_2^-: 0.019±0.005 mg L^{-1}; N-NH_3^+: 0.19±0.25 mg L^{-1}. Measured salinities were closed to expected values (±0.3 ppt) (**Figure 1**).

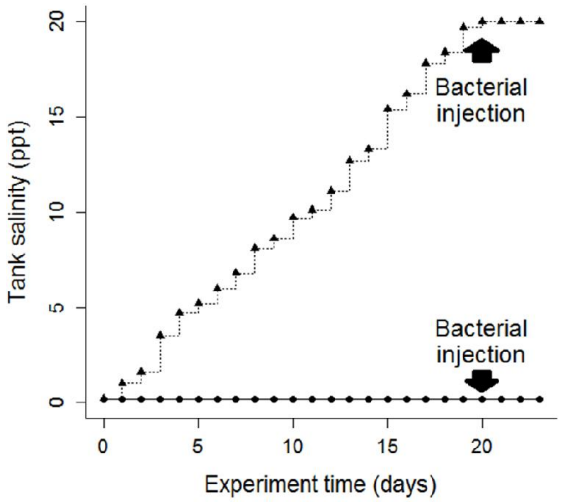


Figure 1: Experimental design of striped catfish multi-stress experiment. Striped catfish were exposed (or not) to increasing salinity during 20 days until 20 ppt, and then to bacterial infection during 3 days.

2.2 Bacterial challenge

Culture of the strain

A virulent strain of *E. ictaluri* (TNA 015, Can Tho University, Vietnam) was cultured on Brain Heart Infusion (BHI) agar (Sigma), at 28°C and pH 7.4. Small transparent and round colonies appeared after 2 days of incubation. The pure colonies were checked throughout Gram staining in light microscopy and shape of the colonies.

Bacteria count

Bacteria were first numbered in a reference bacteria solution with optical density of 0.1 at 590 nm. Quickly, serial exponential dilution (100 to 10^{10}) of 100 µl of bacteria solution were cultured in BHI agar during 48 h at 28°C and the colonies were counted. A method of confirmation using DNA fluorochrome 4'-6'-diamidino-2-phenylindole was used. Serial exponential dilutions (10^4 to 10^8) were incubated 1 h in DAPI, filtrated and counted on black filters under fluorescent microscope. We estimated that a reference *E. ictaluri* solution of DO 0.1 cultured in BHI contained approximately 10^9 CFU ml^{-1} solution.

Lethal dose 50% (LD 50 72 h)

In a preliminary experiment, different doses of bacteria suspended in HBSS were intraperitoneally injected to fish (n = 6 per tank) using an inoculation volume of 0.025ml g^{-1} in order to estimate the LD 50 72 h. The results indicated that, injection of 0.025 ml g^{-1} fish of a bacterial solution containing 10^6 colony forming unit ml^{-1} induced 50 % mortality after 72 h. For the experiment, a bacterial solution containing 10^5 colony forming unit g^{-1} fish was injected.

Confirmation of infection

Using Biolog biochemical identification systems, infection was confirmed on the kidney (100 % probability for *E. ictaluri*) by the BRLFD-CER group (Belgian Reference Laboratory of Fish Diseases-Centre d'Economie Rurale, Aye, Belgium), a laboratory specialized in screening and diagnosis of fish viral, bacterial and parasitic pathologies.

2.3 Osmoregulatory parameters

Plasma osmolality

Plasma osmolality (using100 µl aliquot) was measured with a micro-osmometer (Type 6, Löser Messtechnik, Germany) in duplicates according to the depression of freezing point compared to pure water.

Gill Na^+K^+ ATPase activity

Gill lysates were obtained by homogenizing gill filament for 2 X30 s in ice cold SEI buffer (Sucrose 0.25 M, EDTA 1 mM, Imidazole 50 mM, pH 7.4), containing a protease inhibitor cocktail (Sigma) using sterile potter homogenizer. Main debris were removed by 2 successive centrifugations at 10 000 g during 5 min at 4°C. In duplicates, an aliquot (50 µl) was used to measure Na^+K^+ ATPase activity according to the method of Mc Cormick (1993). One unit of Na^+K^+ ATPase activity represents the consumption of 1 µmole NADH min^{-1} ml^{-1}.

2.4 Statistical analysis

Homogeneity of variances was tested by Levene test and normality was checked by Shapiro-Wilk test. Changes in these parameters were analysed by Student's t-test ($p<0.05$). Graphs and statistical calculation were performed in R. Data are represented as the mean ± SD and tanks were used as the statistical unit (n = 4, 6 fish).

2.5 Label free proteomics

Proteolysis

Proteomics analysis was performed on samples from day 20 and 23. Three biological replicates were performed. The experimental unit was the tank (n=3) and 6 fish from the same tank were pooled together. Kidney samples were grinded in 9M Urea and sonicated. Then, a 10µg amount of the resulting proteins was reduced with DTT 2.8mM (60ºC for 30 min), modified with 8.8mM iodoacetamide in 100mM ammonium bicarbonate (in the dark, room temperature for 30 min) and digested in 2M Urea, 25mM ammonium bicarbonate with modified trypsin (Promega) at a 1:50 enzyme-to-substrate ratio, overnight at 37ºC. An additional second

digestion was done for 4 hours. The resulting tryptic peptides were desalted using C18 stage-tip (Harvards), dried and re-suspended in 0.1% formic acid**.**

Mass spectrometry

The peptides were loaded onto a C18 trap column (0.3 x 5mm, LC-Packings) connected on-line to a homemade capillary column (25 cm, 75 micron ID) packed with Reprosil C18-Aqua (Dr Maisch GmbH, Germany) in solvent A (0.1% formic acid in water). The peptides mixture was resolved with a (5 to 28%) linear gradient of solvent B (95% acetonitrile with 0.1% formic acid) for 120 min followed by 5 min at gradient of 28 to 95% and 25 min at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.15 µl min^{-1}. Mass spectrometry was performed in a positive mode (m/z 350–1800) using repetitively full MS scan followed by high collision induces dissociation (HCD, at 35 normalized collision energy) of the 10 most dominant ions (>1 charges) selected from the first MS scan. A dynamic exclusion list was enabled with exclusion duration of 20s.

Data analysis

The MS raw data were analysed by the MaxQuant software (version 1.5.1.2, http://www.maxquant.org) for peak picking and quantitation, followed by identification using the Andromeda search engine (Cox et al., 2011), searching against the Characiphysae section of the NCBI-NR database (Jan2015, 54767 proteins) with mass tolerance of 20 ppm for the precursor masses and for the fragment ions. Methionine oxidation was set as variable post-translational modifications and carbamidomethyl on cysteine as a static one. Minimal peptide length was set to six amino acids and a maximum of two miscleavages was allowed. Peptide- and protein-level false discovery rates (FDRs) were filtered to 1% using the target-decoy strategy. Protein and peptides tables were filtered to eliminate the identifications from the reverse database and from common contaminants. The MaxQuant software was used for label-free quantitative analysis, based on extracted ion currents (XICs) of peptides enabling quantitation from each LC/MS run for each peptide identified in any of experiments (Cox et al., 2014). Peptides and proteins tables are available in the online supplementary table. Only proteins that were identified with at least two peptides in one of the samples are listed in the online supplementary table. The intensity data were log -2 transformed in order to get a normal distribution. Missing values were replaced with 10. T-Test with Permutation-based FDR, (with 250 randomization, Threshold value=0.05) between freshwater and saline water fish proteins was done using Perseus 1.5. In order to visualize the whole dataset, hierarchical clustering of log2 intensities using Euclidian distances and average linkage was performed in Permutmatrix, a free bioinformatics platform developed by Caraux & Pinloche (2005). Additional analyses on protein abundances such as Principal Component Analysis were performed in R (not shown). In the next step, DAVID (Database for Annotation, Visualization and Integrated Discovery) platform v6.7 was used to enrich functionally-related proteins categories and visualize pathways on KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway maps (Huang et al., 2009). By default, DAVID used the whole set of proteins present in the proteome as the background. Since a complete description of striped catfish proteome is lacking, we needed to unify proteome annotation using the model organism zebrafish (*Danio rerio)* orthologs to ensure that DAVID could handle the associated protein IDs. In addition, the visualization of pathways in KEGG required accurate pathway maps that are only available for zebrafish. GI accession numbers were used for this purpose. This step constituted a significant unspecific filter and resulted in loss of protein because of a lack of correspondences. Then, proteins IDs for *D. rerio* were submitted to DAVID interface in order to perform the enrichment analysis of the ontologies and pathway mapping (EASE score <0.1).

Western blot validation

Three proteins were quantified in duplicates by western blotting analysis. Using sterile potter homogenizer, kidney tissue was homogenized 2X30s in Tris-HCl 50mM , NaCl 150 mM, SDS 0.1%, Triton X-100 1%, apopritin 0.001 mg ml^{-1}, pH.8 (d1:3). Lysates were then sonicated 3X10 s at 45 kHz and 5x1s at 65 kHz on ice and centrifuged at 10 000 g for 10 min to remove main debries. Total protein in the samples were measured by Pierce method using BSA 1 mg ml^{-1} as standard. Then, 20 µg protein of kidney lysates were mixed with DTT 0.5 M 0.1 %, NuPage Lithium Dodecyl Sulfate 1:3 and heated at 90°C for 5 min. Samples were centrifuged at 10 000 g for 5 min to remove debris. Prestained standard (161-0138, Biorad) and 20 µg of kidney proteins were separated on a 4-12% NuPage Novex Bis Tris gel in MOPS during 1h at 150 V and transferred onto PVDF membrane using an electrophoretic transfer system during 2h15 at 0.8 mA cm^{-2} (BioRad). Membranes were blocked in PBST (PBS pH 7.4, containing 0.1% Tween-20) containing 5% skim milk overnight at 4°C. The next day, membranes were rinsed 2X5 min in PBST and probed with anti-peroxiredoxin 1 (Abcam, ab41906) / anti-gelsolin (BD Biosciences, 610412) / anti-hexokinase 2 (Abcam, ab131196) 1:2000 diluted in 2% blocking buffer for 90 min at room temperature under constant agitation. After washing 2X5 min in PBST, membranes were incubated with anti-rabbit IgG-Alkaline Phosphatase antibody (Acris, R1364) / anti-mouse IgG Peroxidase antibody (Amersham, NA931) 1:5000 diluted in 2% blocking buffer for 1 h at room temperature. Membranes were rinsed 2X5 min in PBST and revealed with BCIP/NBT (Sigma) / ECL Plus Western Blotting substrate (ThermoScientific) in Biorad Imaging System. Relative quantification was done by using ImageJ software and compare with MS results. After immunodetection, a loading control was performed following the method of Welinder & Ekblad (2011) in Coomassie Brilliant Blue R-250 (Biorad).

3 Results

3.1 Osmoregulatory capacities of striped catfish

The osmoregulatory response of striped catfish to increasing salinity was investigated through plasma osmolality (**Figure 2 A**) and gill Na^+K^+ ATPase (**Figure 2 B**). Osmolality of catfish raised in freshwater varied from 254 to 271 mosm and gradually increased significantly with increasing salinities to reach on average 370 mosm at 20 ppt on day 20 ($p<0.001$). The bacterial infection did not induce significant changes in plasma osmolality ($p<0.05$). Prior to infection, gill NKA ATPase activity was comprised between 0.32 and 0.64 U mg^{-1} gill min^{-1} in fish maintained in freshwater but significantly increased up to 1.68 ± 0.32 U mg^{-1} gill min^{-1} ($p<0.05$) in salinity treated fish. In infected fish, gill Na^+K^+ ATPase activity remained stable around 0.69 U mg^{-1} gill min^{-1} in freshwater but significantly decreased to 0.38 U mg^{-1} gill min^{-1} in saline water ($p<0.001$).

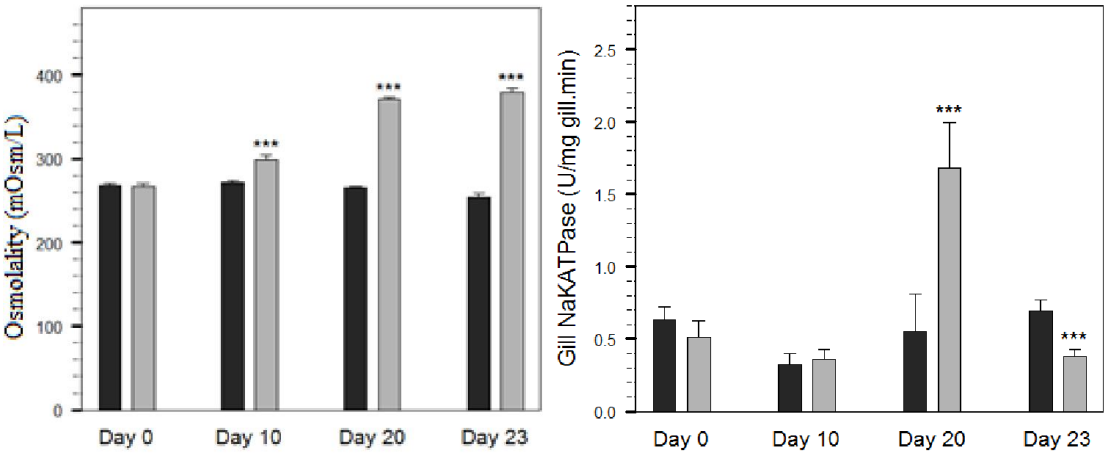


Figure 2: Osmoregulatory responses of striped catfish exposed (or not) to elevated salinity and experimentally infected with *Edwardsiella ictaluri*. Plasma osmolality (A) and gill Na^+K^+ ATPase activity (B) of striped catfish exposed to freshwater (black) and saline water gradient (0-20 ppt) during 20 days (grey) and 3 days post-infection (day 23). The values were presented as the mean ± SD with n=4 (4 tanks, 6 fish/tank) and analysed by Student's t-test. ** $p<0.01$ compared with freshwater fish; *** $p<0.001$ compared with freshwater fish.

3.2 Analysis of kidney proteome

Hierarchical clustering of the differentially expressed proteins

Complete analysis resulted in identification of 2024 proteins, among which 496 proteins showed significant changes in abundance when salinity increased. In order to visualize these differentially-expressed proteins, a hierarchical clustering of the differentially expressed proteins has been performed in figure 3. By cutting the experimental conditions (columns) on the second level, we outlined three clusters, the first cluster (A) included the freshwater fish (freshwater, not infected); a second cluster (B and C) grouped fish raised in saline water, infected or not; a third group (D) contained infected fish raised in freshwater. Triplicates (3 tanks per condition) are clustered together. At the protein level (rows), 4 major clusters appeared up or downregulated. In cluster 1 (5.4 % of the differentially expressed proteins), proteins appeared to be mainly upregulated in infected fish (cluster C and D). Cluster 2 grouped 32.1% of the differentially expressed proteins and appeared to be upregulated in saline water (B and C). Clusters 3 and 4 gathered 62.5 % of the differentially expressed proteins, dominated by a synergic effect of both salinity and infection, with a progressive gradient of expression in response to higher stress intensity.

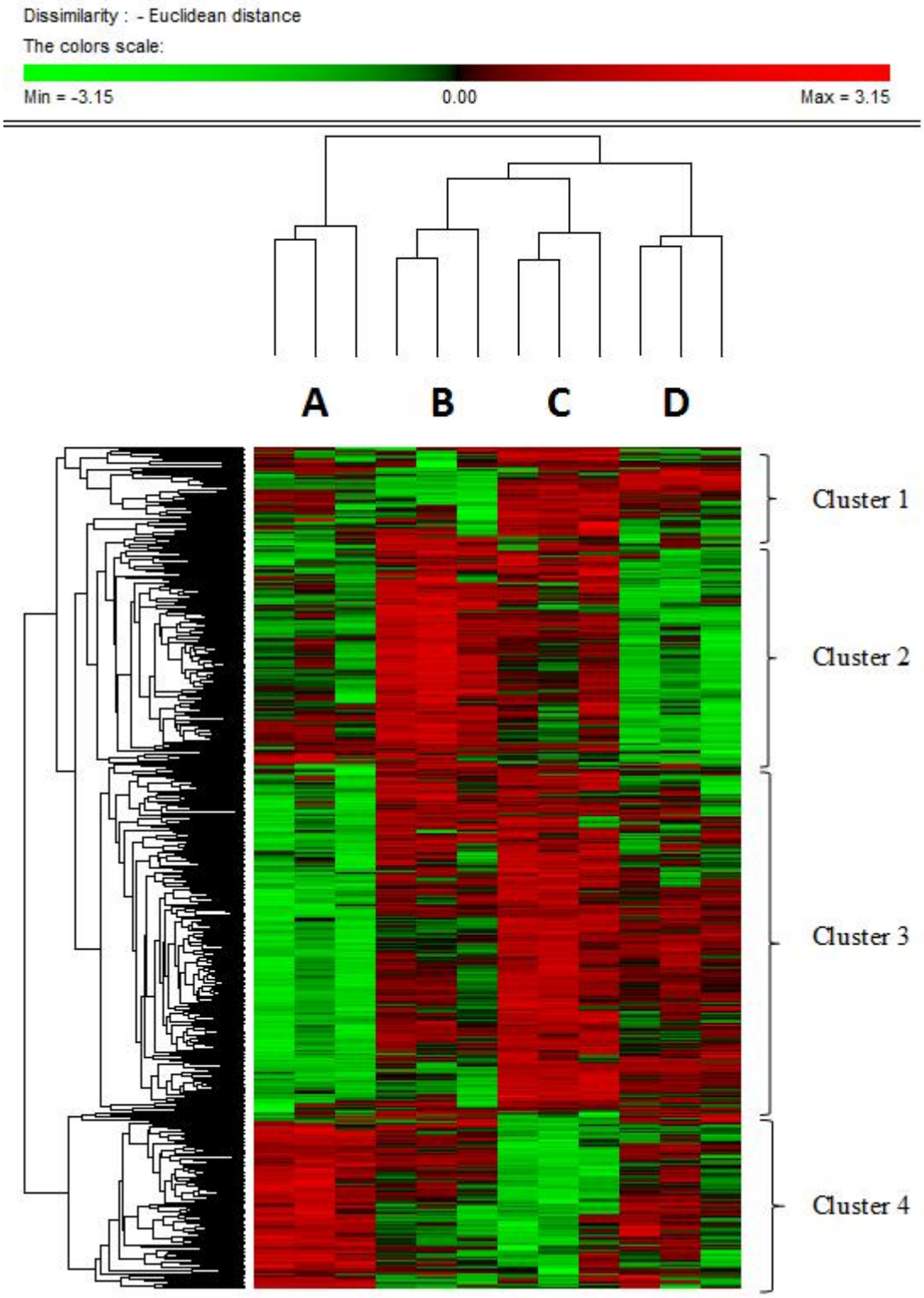


Figure 3: Hierarchical clustering of proteomic data. The heat map displays assemblies of proteins with significant changes in abundance (FDR<0.01) related to salinity increase and/or bacterial infection. Data were obtained following T-Test, Z-score normalization of rows (proteins) in log2 intensities using Euclidian distances and average linkage. Intensity ranges from low (green) to high (red). A: Freshwater, not infected; B: Saline water, not infected; C: Saline water, infected; D: Freshwater, infected.

Pathway analysis of the differentially expressed proteins

A pathway analysis of the whole dataset was performed to isolate the major specific functional pathways. Among the 496 differentially expressed proteins, 395 proteins were processed by DAVID platform and 16 main pathways were outlined (EASE score<0.1). **Table 1** shows the results obtained from the pathway enrichment analysis approach in DAVID platform. **Figure 4** represents an example of major functional pathways resulting from DAVID analysis in KEGG database, particularly the ribosomal subunits.

The major enriched pathway involved ribosomes and gathered 55 structural ribosomal components of the small 40 S and large 60S subunits of ribosome. All ribosomal proteins were upregulated with salinity and infection (cluster 3 in the hierarchical clustering).

The pathway analysis mainly put in evidence enzymes involved in energy production, including glycolysis (e.g. hexokinases 1-2, pyruvate dehydrogenase α/ß, pyruvate dehydrogenase α/ß), citrate cycle (e.g. isocitrate and malate dehydrogenase, citrate synthase), fatty acid β oxidation (e.g. acetyl-CoA acetyltransferases, long chain and very long chain acyl-CoA dehydrogenases), oxidative phosphorylation (complex I, III, IV and V) and ketone bodies synthesis (e.g. acetyl-CoA acetyltransferases 1-2). These proteins were mainly stimulated by salinity (cluster 2). An exception has been observed for perixosomal enzymes (e.g. peroxisomalacetyl-coA oxidases, 3-hydroxyacyl-CoA dehydrogenase) which at the opposite highly decreased acetyl-coA oxidases, 3-hydroxyacyl-CoA dehydrogenase) which at the opposite highly decreased proportionally to saline stress intensity (cluster 4).

In addition to glycolysis and gluconeogenesis pathways, other pathways were related to carbohydrate metabolism. The galactose, fructose and mannose metabolism were over expressed during infection and salinity (cluster 3). The aminosugar and nucleotide sugar metabolism included glycosyl transferase enzymes downregulated with salinity and infection (cluster 4) and the glutamine-fructose-6-phosphate transaminase 1, strongly induced in infected fish (cluster 1).

Pathway analysis highlighted many enzymes involved in amino acid metabolism. Biosynthesis of valine, leucine, isoleucine was enhanced with salinity and infection (cluster 3) while their degradation increased in saline condition (cluster 2). Tryptophan metabolism included enzymes involved in niacin metabolism (downregulated with salinity and infection, cluster 4) and enzymes linked to energetic metabolism (enhanced with salinity and infection, cluster 3). In glycine, serine and threonine metabolism, salinity and infection enhanced the abundance of enzymes involved in degradation of glycine and threonin (cluster 2, 3) while decreased the abundance of the sarcosine dehydrogenase, involved in glycine biosynthesis and the D-amino acid oxidase, which links glycine to the glyoxylate cycle (cluster 4). Phenylalanine metabolism mainly grouped ubiquitous enzymes (e.g. monoamine oxidase, aspartate aminotransferase), upregulated in saline condition (cluster 2).

Enhanced protein turnover with salinity and infection was outlined by the increase in abundance of 8 polypeptides of the core (subunits α 1-3-5 and ß 2-5-7) and regulatory (216S and 26S subunits) particles of the proteasome complex (cluster 2 and 3).

In porphyrin metabolism, DAVID outlined 8 key proteins in heme metabolism and iron transport. Infection induced an increase in transferrin, transferrin precursor, ceruloplasmin and ceruloplasmin precursor (cluster 1) while salinity was responsible for higher abundance of phorphobilinogen deaminase (third step of heme biosynthesis) and ferrochelatase (terminal of heme biosynthesis) (cluster 2). In heme catabolism, the stress-response protein heme oxygenase 1 increased gradually with salinity and infection (cluster 3) whereas the abundance of biliverdin reductase was enhanced by salinity (cluster 2). One protein involved in extracellular sequestration of haemoglobin, the haptoglobin, was not detected by DAVID.

Table 1: Significantly enriched KEGG pathways retrieved by DAVID platform for 395 differentially expressed proteins. This group represented proteins of striped catfish *Pangasianodon hypophthalmus* differentially expressed ($p<0.05$) following high saline stress gradient (0-20 ppt) in infected fish, or not (EASE score<0.1). Count: Proteins involved in the pathway; p-value (EASE score): to examine the significance of protein-term enrichment with a Fisher's exact test; Enrichment: measure the magnitude of enrichment; Benjamini: correct enrichment p-values to control family-wide false discovery rate.

Term	Count	P value	Enrichment	Benjamini
Ribosome	55	4.0 E-48	11.4	3.7 E-46
Energy production				
Glycolysis / Gluconeogenesis	15	3.7 E-6	4.4	1.2 E-4
Citrate cycle	13	7.8 E-8	7.1	3.6 E-6
Fatty acid metabolism	9	3.2 E-4	4.9	5.0 E-3
Oxidative phosphorylation	14	2.1 E-2	2.0	1.3 E-1
Synthesis and degradation of ketone bodies	4	5.0 E-3	10.3	4.1 E-2
Carbohydrate metabolism				
Galactose metabolism	5	4.6 E-2	3.6	2.2 E-1
Fructose and mannose metabolism	7	2.1 E-2	3.1	1.2 E-1
Amino sugar and nucleotide sugar metabolism	9	3.3E-3	3.5	3.7 E-2
Amino acid metabolism				
Valine, leucine and isoleucine biosynthesis	4	3.2 E-3	7.5	4.2 E-2
Valine, leucine and isoleucine degradation	11	5.4 E-5	4.8	1.3 E-3
Tryptophan metabolism	8	5.6 E-2	2.8	2.5 E-1
Glycine, serine and threonine metabolism	6	2.3 E-2	3.6	1.3 E-1
Phenylalanine metabolism	4	9.5 E-2	3.6	3.5 E-1
Proteasome	8	2.5 E-2	2.7	1.3 E-1
Iron metabolism				
Porphyrin and chlorophyll metabolism	8	7.3 E-2	3.1	3.0 E-1

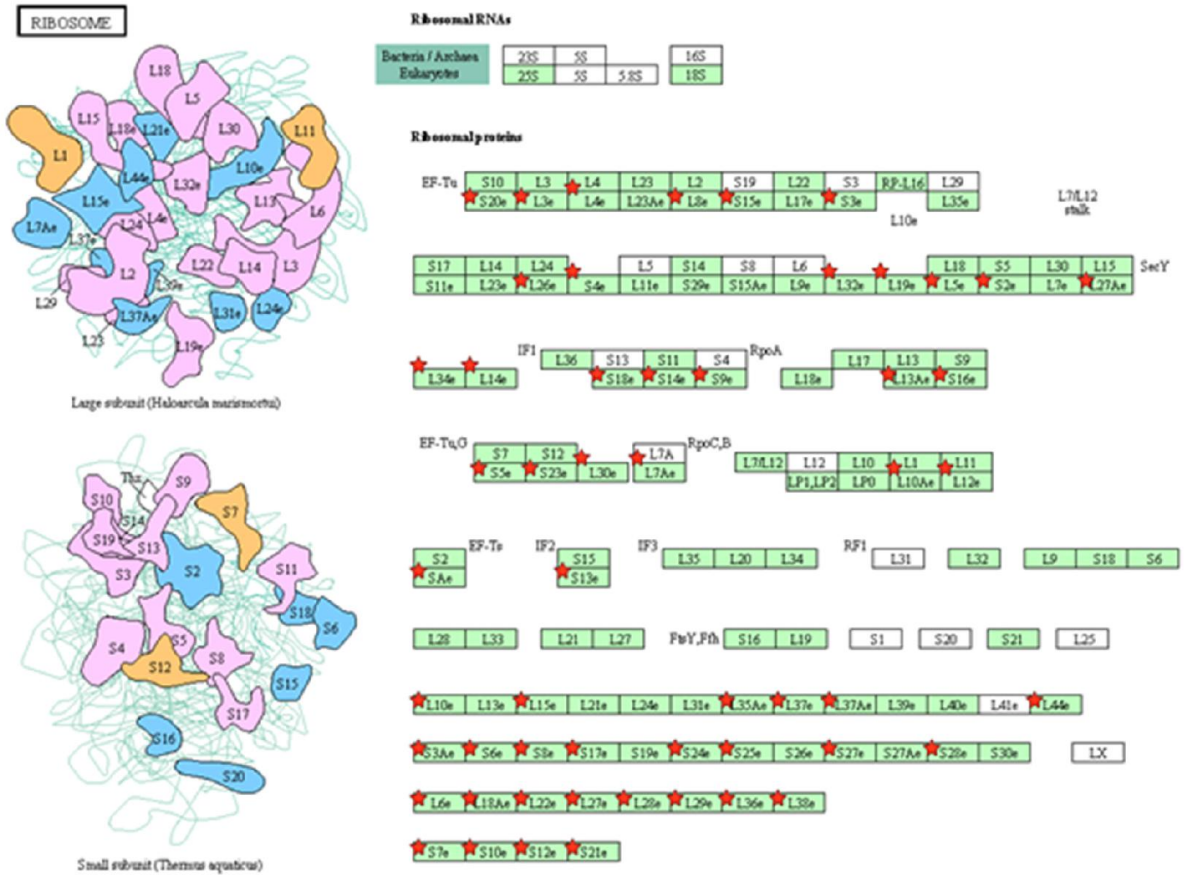


Figure 4: Examples of enriched pathways resulting from DAVID analysis in KEGG database. The ribosomal proteins (A) (by homology with *Danio rerio* database) were significantly enhanced following saline conditions in healthy or infected fish. Red stars represent differentially expressed proteins.

Functional categories of the differentially expressed proteins

An enrichment analysis was conducted on ontologies in DAVID on protein GI identifiers. On the 3 main categories of the GO terms represented, 46.3 % belongs to metabolic processes, 46.1% to cellular processes and 7.6 % to response to external stimuli (i.e. stress). **Table 2** provides a summary of the main functional categories obtained in DAVID (EASE score<0.1).
In nucleic acid metabolism, nucleotides-binding proteins and ribonucleoproteins are the most enriched categories and mostly belong to cluster 3. The first category gathered diverse proteins involved in transcription and splicing, nucleic acid transport and nucleic chaperones. The second category gathered several ribonucleoproteins including ribosomal proteins, small and heterogeneous nuclear ribonucleoproteins.
The next most represented functional categories was protein metabolism. As in the pathways analysis, DAVID outlined 8 polypeptides from the proteasome complex as well as several polypeptides implicated in amino acid biosynthesis (**Table 2**). In addition, DAVID outlined proteins involved in protein biosynthesis such as translation initiation factors, elongation factors and t-RNA ligases. All translation factors displayed increasing abundance in relation to stressors association (cluster 3) while ligases were dispersed into clusters 2 and 3. Protein transport included vesicle mediated and intracellular transporters (e.g. Sec 23A, SAR-1A, vacuolar transporter, coatomer γ2) as well as mitochondrial transporters (e.g. inner membrane translocases, carnitine carrier protein). Proteins involved in transport were dispersed throughout clusters 1, 2, 3 but were completely absent from cluster 4. The last functional category related

to protein metabolism grouped chaperones involved in protein folding (HSP 10, 60, 70, 90, T complex subunits and calnexin), that were generally enhanced with salinity and infection (cluster 3). Some of them were implicated in stress response, including more specific proteins such as fibrinogen ß and γ, heme oxygenase, lactate dehydrogenase and anti-oxidizers (i.e. peroxiredoxins 1-6, catalase, glutathione peroxidase and superoxide dismutase).
The next functional category included enzymes involved in generation of primary metabolites and energy. It grouped the same enzymes as in pathway analysis (**Table 2**).
Three main cellular components were represented in our results. The two most important were the cytoskeleton and the mitochondria constituents. For cytoskeleton, the abundance of intermediate filaments of keratin, myosin and desmin decreased with salinity and infection (cluster 4). In the opposite, other polypeptides associated with actin filament organization and structure (i.e. gelsolin, actin, calponin, glia maturation factor ß, profilin, plectin, filamin B, rho GTPase) were increasing with salinity and infection (clusters 2 and 3). For mitochondria, proteins included mitochondrial chaperones, enzymes involved in energy production, inner membrane transporters and others (e.g. apoptosis inducing factor 1, fission process protein 1, clustered mitochondria protein). Nearly all mitochondrial proteins were exclusively stimulated by salinity (cluster 2). In addition, DAVID outlined only 5 peroxisomal proteins, all decreasing with salinity and infection (cluster 4).
Pertaining to the extracellular matrix, 9 types of collagen α chain and associated-protein decorins decreased with salinity and infection (cluster 4). Collagen chains and decorins were gathered in a same branch of the hierarchical clustering dendrogram (**Figure 3**).
The last functional category included proteins entailed in ion homeostasis. The abundance of subunits α and ß Na^+ K^+ ATPase, solute carrier family 12 ($Na^+/K^+/2Cl^-$ transporter), calcium-binding protein p22 (Na/H exchanger) increased during exposure to elevated salinity (cluster 2) whereas the abundance of the solute carrier family 4 (Cl^-/HCO_3^- exchanger) decreased in fish held at high salinity (cluster 4). Furthermore, the lysosomal V-type ATPase increased when salinity and infection were combined with each other (cluster 3). The last functional group clustered calcium-binding proteins. Most of calcium-binding proteins were upregulated in hyperosmotic conditions although annexins, calpain regucalcin and spectrin α and ß chain were regulated also with infection.

Table 2: Functional categories enrichment analysis by DAVID platform for 395 differentially expressed proteins. (EASE score<0.1). This group represents proteins of striped catfish *Pangasianodon hypophthalmus* differentially expressed ($p<0.05$) following high saline stress gradient (0-20 ppt) in healthy or infected fish. Count: Proteins involved in the pathway; p-value (EASE score): to examine the significance of protein-term enrichment with a Fisher's exact test; Enrichment: measure the magnitude of enrichment.

Term	P value	Enrichment
Nucleic acid metabolism		
Nucleotide-binding protein	3.8e-23	66.0
Ribonucleoprotein	1.93 ^{E}-14	10.5
Protein metabolism		
Proteasome	6.2 ^{E}-4	5.4
Protein biosynthesis	2.32^{E}-12	5.3
Amino acid metabolism	1.6e-5	3.2
Chaperone	3.25^{E}-9	7.2
Protein transport	0.02	2.9

Response to stress	0.04	5.1
Generation of precursor metabolites and energy	7.1E-9	1.5
Cellular component		
Cytoskeleton and actin binding protein	7.4e-2	2.2
Mitochondrion	0.009	2.5
Peroxisome	4.3e-2	110.0
Extracellular matrix		
Collagen	6.8e-11	250.0
Ion homeostasis		
Ion transport	4.8e-7	3.0
Calcium ion binding	4.2e-12	16.0

Western blot validation

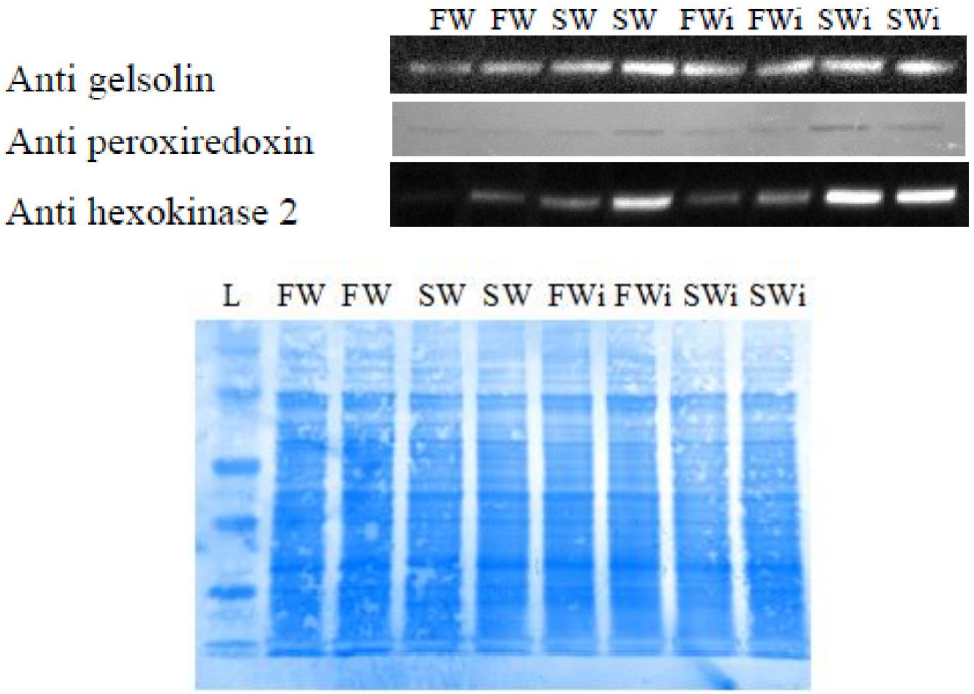


Figure 5: **Western blot validation**. Detection and relative quantification of gelsolin, peroxiredoxin 1 and hexokinase 2. The bands represent detected protein of striped catfish *Pangasianodon hypophthalmus*) revealed in chemiluminescence (gelsolin, hexokinase 2) or colorimetry (peroxiredoxin 1) by Biorad Imaging System. Whole protein stain revealed in Coomassie Brilliant Bleu R 250. L: Ladder, FW: Freshwater, not infected; SW: Saline water, not infected; FWi: Freshwater, infected, SWi: Saline water, infected.

A unique band was detected for gelsolin around 90 kda, peroxiredoxin 1 around 25 kda and hexokinase 2 around 75 kda (**Figure 5 A**). Quantification analysis by Image J indicated overexpression peroxiredoxin 1 by respectively 1.32, 1.28 and 2.80 fold in saline water, freshwater infected and saline water infected compared to freshwater while in LFQ peroxiredoxin 1 increased respectively by 1.46, 1.45 and 1.97 fold. Gelsolin increased by 1.12 to 1.91 fold when salinity increased while in LFQ gelsolin increased by respectively 1.06 to 1.24 fold. To finish, hexokinase 2 abundances were 5.00, 2.50 and 9.66 fold respectively in saline water, freshwater infected and saline water infected compared to freshwater. In LFQ,

hexokinase 2 respectively increased by 1.50, 4.33 and 9.41 fold. Whole protein stain revealed equal protein loads (**Figure 5 B**).

3.3 Susceptibility to ESC

Mortalities were significantly higher at 20 ppt compared to freshwater ($p<0.05$). No mortalities were observed in freshwater fish 72 h post injection while mortalities reach 67±14 % of the fish at 20 ppt.

4 Discussion

Specific response of striped catfish to hyperosmotic stress and renal excretion capacity

Plasma osmolality values were similar to those reported by Eckert et al.,(2001) and Phuc et al.,(2014) for the channel catfish and striped catfish respectively. At 10 ppt (270 mosm), plasma osmolality equilibrated with media salinity. At 20 ppt (540 mosm) plasma osmolality also increased but to a level lower than that of the environment (370 mosm ± 3 mosm), meaning that the fish may excrete a part of the ionic overload, as indicated by the increasing gill Na^+K^+ ATPase. Therefore, gill electrolyte clearance in striped catfish may be insufficient to cope with the increased salt load. The osmotic response of striped catfish is similar to that observed in channel catfish. Transfer of channel catfish from freshwater to seawater induced an elevation in plasma osmolality and the production of small volume of urine hypotonic to plasma (Norton & Davis, 1977; Eckert et al., 2001). Decrease in gill vascularisation is frequent in fish infected by *E. ictaluri*, including striped catfish and may be responsible for gill ion transport collapse (Yuasa et al., 2003; Shigen et al., 2009).

Ion fluxes in osmoregulatory organs are driven by highly specialized chloride cells (also referred as to mitochondrion-rich cells and ionocytes), characterized by abundant mitochondria and ion transporters and extensive basolateral tubular membrane (Evans et al., 2005). Chloride cells have been mainly described in gill epithelium (Christensen et al., 2012; Kültz et al., 2013) while renal chloride cells have been poorly investigated. In the euryhaline European sea bream *Sparus aurata*, chloride cells associated with active Na^+K^+ activity expression were discovered in the collecting duct of kidney (Nebel et al., 2005). In our study, specific response to salinity stressor was mainly characterized by up regulation of mitochondrial proteins (i.e. HSP, transporters, energy, branched chain amino acid degradation) and ion transporters. The overall high consistency of upregulation of mitochondrial proteins agrees with an increase of mitochondria. Moreover, key proteins involved in ion transport across epithelium of chloride cells including vacuolar-type H^+ ATPase, Na^+ K^+ ATPase and $Na^+/K^+/2Cl^-$ transporter are also upregulated (Esaki et al., 2009; Christensen et al., 2012). In proteomics, activation of the energetic metabolism associated with modulation of osmoregulation system has already been described after hyperosmotic shock in the gill of number of euryhaline species such as European seabass, Mozambique tilapia *Oreochromis mossambicus* and ayu *Plecoglossus altivelis* while information scarce on kidney osmoregulation (Fiol et al., 2006; Gillardin et al., 2012; Ralston-Hooper et al., 2013). In response to hyperosmotic stress, the striped catfish may increase the size or the number of chloride cells in kidney proximal and distal tubules, in order to increase the excretory capacity of the kidney while minimizing water loss (Beyenbach, 1995; Beyenbach, 2004). However, inefficient gill excretory mechanisms may induce inadequate salt excretion and may fail to decrease plasma osmolality down to freshwater values.

Regarding porphyrin metabolism, salinity increased the abundance of enzymes involved in heme biosynthesis. Hemoproteins are ubiquitous and involved in many biological pathways including the mitochondrial electron transport chain in order to produce energy. Porphyrin

metabolism is also characterized by upregulation of biliverdin reductase a, a major antioxidant cytoprotectant. In our study, salinity induced upregulation of many antioxidizers. Increase in antioxidant defences may counteract the deleterious side effects of reactive oxygen species (ROS) production by the mitochondrial electron transport chain and monoamine oxidase.

The number of calcium-binding proteins differentially expressed in this study, particularly in fish exposed to saline water. In animals, it has been recognized that intracellular calcium may be a key messenger in response to hyperosmotic stress through regulation of permeability control and cytoskeleton reorganization (Chang et al., 2004; Fiol & Kümtz, 2007; Prodocimo et al., 2007; Papakostas et al., 2012). Infection may have an important effect on calcium sensing proteins too. The strongest effect observed in our study is related to regucalcin protein, which regulates calcium homeostasis and is known to exert a suppressive effect in cell proliferation (Yamaguchi et al., 2011; Yamaguchi et al., 2014). Saline water and infection downregulated regucalcin respectively by 2.5 and 2.8 fold. When both types of stressors were applied simultaneously, the suppressive effect reach abundance 16 fold inferior than that in freshwater, not infected. In fish, downregulation of regucalcin have already been described 72h after bacterial infection in rainbow trout (*Oncorhynchus mykiss*) and maraena whitefish (*Coregonus maraena*) (Verleih et al., 2013).

Salinity stressor slightly decreased fish immune defences: iron metabolism and complement pathway

Iron metabolism is known to play a central role in host-pathogen interaction. Indeed, pathogen multiplication inside the host depends on the iron uptake ability of the organism (Ratledge, 2000). Increasing the abundance of high-affinity iron binding proteins may limit the concentration of free circulating iron thereby reducing its availability for the pathogen. In several species of catfish, it has been reported that the most highly upregulated group of genes after *E. ictaluri* infection is involved in iron homeostasis and include ceruloplasmin, transferrin, haptoglobin and ferritin (Peatman et al., 2007; Peatman et al., 2008). In our study, infection upregulated by 2 to 4 fold iron transport proteins while salinity downregulate these proteins by 1.3 to 2.0 fold. By decreasing iron transporters, stress response to salinity may counteract the strategy of the fish to reduce free iron availability for the pathogen and thus may promote its multiplication inside the host.

Fish subjected to saline water displayed down regulation of complement factors C3 (1.3 fold) and B/C2A (1.9 fold) before and after infection as well as a strong up regulation of factor D (6.6 fold) only before infection. Wap65-2 precursor varied in the same way as complement factors C3, decreasing by 2.3 fold in saline water. Wap65-1 precursor was also detected in our analysis but showed no differential expression, neither in saline water nor after bacterial infection. In fish, Wap65, particularly Wap65-2, is upregulated in immune response (Peatman et al., 2007; Shi et al., 2010), heavy metal exposure (Aliza et al., 2008) and temperature acclimation (Sha et al., 2008). Up regulation of Wap65 genes has already been described in channel catfish and blue catfish (*Ictalurus furcatus*) after infection with *E. ictaluri* (Aliza et al., 2008; Sha et al., 2008). In our study, infection increased the abundance of Wap65-2 by 3.4 fold in freshwater and by 4.2 fold in saline water, following the trend of complement factor C3. The biological significance of Wap65 is unknown although potential role in anti-inflammatory responses has been recently suggested (Diza-Gonzalez et al., 2014). Correlation of C3 component and Wap65-2 in the clustering of the whole dataset suggests that Wap65 may interact with the complement pathway. Further, recent studies of specific protein-protein interaction in ayu put in evidence a specific interaction between C3 and Wap65 (Shi et al., 2010).

General stress response: the multi-stress approach

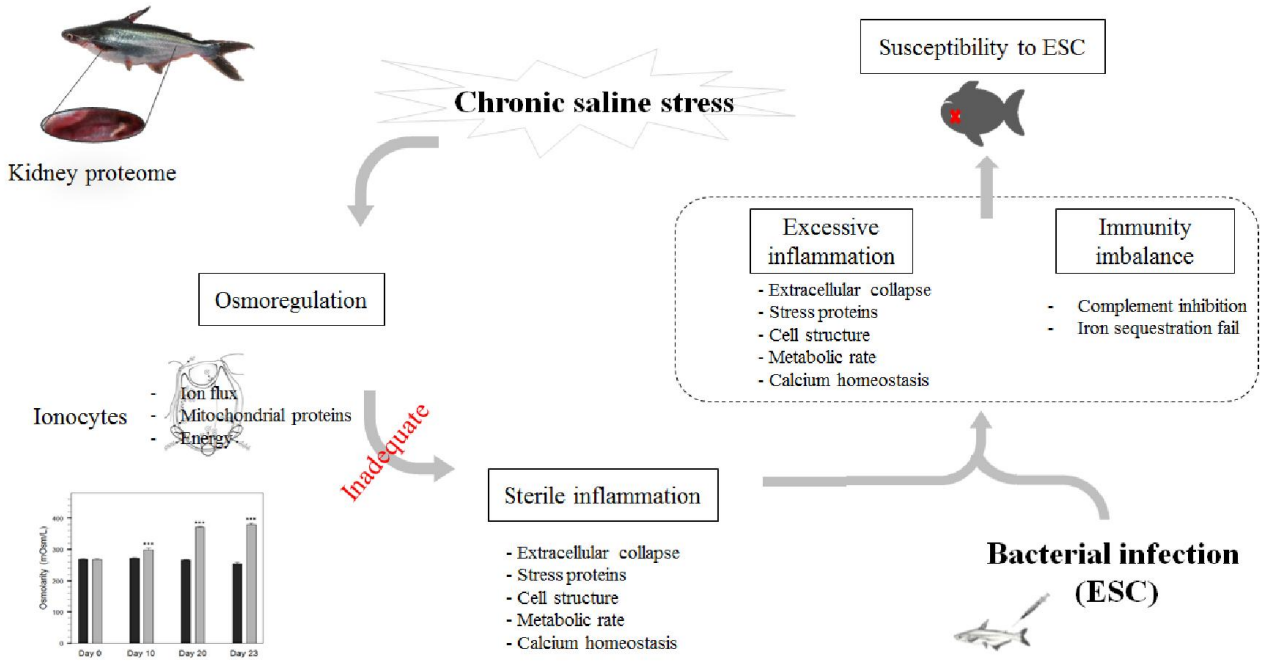
In aquatic organisms exposed to environmental stressors, up regulation of nucleic acid, protein and energy metabolisms has been largely discussed in transcriptomic and proteomic studies (Boutet et al., 2006; Fiol et al., 2006; Kalujnaia et al., 2007; Papakostas et al., 2012). In our study, the multi-stress approach highlighted a correlation between stressors combination and up regulation of a growing set of proteins involved in general metabolic pathways from gene regulation to downstream cellular responses. In particular, HNRPs are known to modulate gene expression during physical or chemical cellular stress (Biamonti et al., 2009). HNRPS are targeted by small ubiquitins related modifiers, which are involved in a wide range of biological processes, particularly in response to environmental stressors such as osmotic shock, hypoxia, hyperthermia or toxicity (Tempé et al., 2008). At the opposite, a decrease of proteins involved in peroxisomal fatty acid ß-oxidation was observed. In fatty acid ß-oxidation, mitochondria oxidize small, medium and long-chain fatty acid to provide energy for several cellular processes (Reddy & Hashimoto, 2001; Poirier et al., 2006). In turn, peroxisomal ß-oxidation is streamlined toward specific carboxylic acids including branched-chain, very long-chain fatty acid, dicarboxylic acids, prostanoids and bile acid intermediates (Reddy & Hashimoto, 2001; Poirier et al., 2006). Moreover, the net energy balance is much lower and a significant part of the energy is lost towards production of ROS by products. In aquatic organisms, peroxisomal proliferation may occur when animal faces organic or toxic xenobiotics (Carajaville et al., 2003) while influences of biotic factors or pathogenic attacks have been poorly investigated. Our study suggests that salinity and infection by *E. ictaluri* may decrease the number or size of peroxisomes. It may be possible that, in stressful condition, fish favoured mitochondrial ß-oxidation to the detriment of peroxisomal ß-oxidation to reduce ROS formation and increase the net energy budget.

In the functional clustering, a cluster relative to general stress response was identified. This cluster gathered many molecular chaperones, fibrinogen ß and ɣ and stress enzymes such as heme oxygenase and lactate dehydrogenase. Stress-related changes in chaperones and chaperonins abundance were largely debated by other authors (Kültz et al., 2013; Baruah et al., 2010). Indeed, it has been recognized that a cellular stress response induced up regulation of HSC to produce newly formed HSP – the latter being involved in a large panel of responses including immune function and proteome integrity (Roberts et al., 2010). Heme oxygenase (HO) is the rate limiting enzyme in the degradation of heme. In fish, upregulation of HO has been observed during hypoxia (Wang et al., 2008) and exposure to environmental pollutants (Ariyoshi et al., 1990). In mammals, HO is known to increase the glomerular filtration rate and promote sodium and fluids absorption (Nath et al., 2006). Moreover, HO is involved in cytoprotective response and its products have a vasorelaxant, anti-inflammatory and anti-apoptotic effect during kidney acute injury or ischemia (Nath et al., 2006). To our knowledge, this study is the first to describe the upregulation of heme oxygenase during hyperosmotic stress and infectious disease. As suggested by their upregulation in response to a large panel of stressors, molecular chaperones and HO may be key cytoprotective molecules related to the general stress response syndrome.

During cellular stress, eukaryotic cells are able to remodel and reinforce their cytoskeleton in order to withstand the physical challenge. In our study, salinity and infection induced overproduction of actin polymerizing and depolymerizing proteins and regulators (i.e. Rho family small GTPases and the actin-related 2/3 signalling complex (Arp 2/3)) while lowered the abundance of spectrin scaffold, intermediate filaments and extracellular matrix components. Similarly, facing hyperosmotic environments, Mozambique tilapia increased the abundance of gelsolin and annexin A11 while European seabass and European eel *Anguilla anguilla* downregulated keratin and collagen filaments (Fiol et al., 2006; Boutet et al., 2006;

Kalujnaia et al., 2007). In Chinese Hamster Ovary cell line and Dyctiostelium cells, migration of F-actin to periphery allows the cell to withstand with extensive cell shrinkage induced by the hyperosmotic pressure (Aizawa et al., 1999; Di Ciano et al., 2006). This process is mainly mediated by Rho family small GTPases and Arp 2/3 complex (Koyama et al., 2001; Di Ciano et al., 2006). Therefore, in our study, the physical stress encountered by the cell may induce the breakdown of the spectrin scaffolding and central cytoskeleton network in order to rebuilt peripheral filaments below the membrane, ensure cell shape and volume integrity and create a barrier against *E. ictaluri* invasion. In addition, salinity and infection induced overproduction of plectin and filamin, key filament binding proteins involved in cell structure organization. First, these proteins ensured the connection of the cytoskeleton to the membrane and may link the cytoskeleton to extracellular signalling machinery (Flanagan et al., 2001; Osmanagic-Myers et al., 2014). Then, it is currently thought that they act by increasing the stiffness of filaments by introducing orthogonal cross-links to create an organized three-dimensional network (Osmanagic-Myers et al., 2014). To finish, induction of Arp 2/3 and capping proteins such as gelsolin combined with the breakdown of the extracellular matrix have been associated with protrusive forces such as lamellipodium or podosomes, enhancing cell motility and migration in response to wound healing ((Mejillano et al., 2004; Mazur et al., 2010).

The downregulation of proteins involved in retinal metabolism (i.e. retinol binding protein 4-A (RBP 4-A), retinol dehydrogenase 3 and aldehyde dehydrogenase 8-A1) particularly draws our attention. Indeed, the RBP 4-A, involved in vitamin A trafficking, was downregulated up to 13.0 fold in infected fish in saline water. Interestingly, downregulation of retinol binding proteins in fish was also outlined in intestine and kidney proteome during hyperosmotic stress[16,41,52]. In addition, in human cells, ultraviolet radiation reduced intracellular nuclear retinoid receptors (RARs), cellular RBP II and retinol 4-hydroxylase[71]. Retinoic acids are known to play essential and pleiotropic roles in various biological functions including innate and adaptive immunity (Iwata et al., 2004; Mora et al., 2006), energy homeostasis (Acin-Perez et al., 2010), morphogenesis (Prat et al., 2014; Rydeen et al., 2015), ossification (Gudas, 2012), apoptosis and cell differentiation (Gudas, 2013; Das et al., 2014). In mouse, RARs have been detected in the collecting duct in renal principal and intercalated cells, suggesting that retinoic acid may play a specific role in these two specialized cells (Wong et al., 2011). Lee et al.(2000) hypothesized that cellular stress may decrease the cellular retinol metabolism in epithelial cells and that the mitogen-activated protein kinase 4 may have a role in this suppression. Nevertheless, the relation between stressors and the retinol metabolism remains unclear and sets the framework for future investigation.



5 Conclusions

The present study provides novel insights into the molecular mechanisms that promote remodelling of kidney epithelium in stenohaline fish exposed to environmental salinity changes. Moreover, a multi-stress approach has been applied to study the cross responses between osmoregulatory and immune pathways. On one hand, hyperosmolarity and infection may act synergically to activate general stress responses including higher metabolic rate, overproduction of molecular chaperones and stress enzymes and reinforcement of the cell structure. On the second hand, results showed that hyperosmolarity may also counteract several fish immune strategies such as iron-sequestration and complement activation.

Decrease in survival associated with overproduction of DAMPS (damage associated molecular patterns) and extracellular matrix collapse in fish exposed to saline water lead to the hypothesis that chronic exposure to hyperosmotic stress might induce excessive sterile inflammation in kidney tissue and impair the immune response during bacterial infection. In mammals, it has already been suggested that continuous release of damage associated molecular patterns (e.g. typically HSPs, S100 calcium-binding proteins and purine metabolites) following prolonged exposure to the detrimental agent lead to excessive inflammatory response and severe immunopathological conditions that may end up to tissue damage and death (Chen & Nunez, 2010).

However, to study the proteome response does not provide any information neither on protein activity nor on protein localization and cell movements. Reorganization of kidney epithelium and intracellular cell movements should be validated for example in histology or fluorescent microscopy. Protein abundances should be associated with activity quantification according to specific protein activity analysis. Further, a priori analysis such as targeted proteomics or western blotting may be useful to confirm low-certitude protein abundances.

Further, the complexity of whole proteomes exceeds the analytical capacity to isolate low-abundance proteins such as cytokines and chemokines, which have not been detected in our study. Then, the depletion of high abundance proteins by, for example, subcellular fractionation or immunoaffinity column, may be useful to deplete very high-abundance proteins such as cytoskeleton proteins and enrich low-abundance proteins of interest such as proteins involved in the immune response.

Acknowledgements: We are grateful to the staff of CEFRA-Tihange (ULG) and particularly Christian Prignon for providing striped catfish breeders and useful advices in catfish rearing, the University of Can Tho for providing the virulent bacteria strain and Christian Didembourg in URBM (University of Namur) for his help during bacterial strain culture and flow cytometry analysis. Thanks are due to FRIA (Fonds de la Recherche dans l'Industrie et l'Agriculture, Wallonia-Brussels Federation) for providing a PhD grant to M.S. We thank the associate editor Ingrid Miller and the 2 reviewers for their very insightful and constructive comments.

References

Acin-Perez, R., Hoyos, B., Zhao, F., Vinogradov, V., ., Harris, R.A., Leitges, M., Wongsiriroj, N., Blaner, W.S., Manfredi, G., Hammerling, U. Control of oxidative phosphorylation by vitamin A illuminates a fundamental role in mitochondrial energy homeostasis. *FASEB J.* 2010, *24,* 627-636.

Aizawa, H., Katadae, M., Maruya, M., Sameshima, M., Murakami-Murofushi, K., Yahara I. Hyperosmotic stress-induced reorganization of actin bundles in Dictyostelium cells over-expressing cofilin. *Genes Cells* 1999, *4,* 311–324.

Aliza, D., Ismail, I., Kuah, M., Shu-Chien, A., Tenkgku, M. Identification of Wap65, a human homologue of hemopexin as a copper-inducible gene in swordtail fish*, Xiphophorus helleri. Fish Physiol. Biochem.* 2008, 34, 129-138.

Ariyoshi, T., Shiiba, S., Hasegawa, H., Arizono, K. Effects of the environmental pollutants on heme oxygenase activity and cytochrome P-450 content in fish. *Bull. Environ. Contam. Toxicol.* 1990*, 44,* 189-196.

Baruah, K., Ranjan, J., Sorgeloos, P., Bossier, P. Efficacy of heterologous and homologous heat shock proteins 70s as protective agents to *Artemia franciscana* challenges with Vibrio campbelii. *Fish Shellfish Immun.* 2010, *29,* 733-739.

Beyenbach, K.W. Kidney sans glomeruli. *Am. J. Physiol. Renal. Physiol*. 2004, *286*, F811-827.

Beyenbach, K.W. Secretory electrolyte transport in renal proximal tubules of fish. In, Cellular and Molecular Approaches to Fish Ionic Regulation*,* edited by Wood CM and Shuttleworth TJ. San Diego, CA, Academic 1995, *85*-105.

Biamonti, G., Caceres, J.F. Cellular stress and RNA splicing. *Trends Biochem. Sci*. 2009, *34*, 146–153.

Boutet, I., Long Ky, C.L., Bonhomme, F. A transcriptomic approach of salinity response in the euryhaline teleost, *Dicentrachus labrax*. *Gene* 2006, *379*, 40-50.

Cajaraville, M,. Cancio, I., Ibabe, A., Orbea, A. Peroxisome proliferation as a biomarker in environmental pollution assessment. *Microsc. Res. Tech*. 2003*, 61,* 191-202.

Caraux, G., Pinloche, S. Permutmatrix, a graphical environment to arrange gene expression profiles in optimal linear order. *Bioinformatic*s 2005, *21,* 1280-1281.

Chang, W.H., Shoback, D. Extracellular Ca2+-sensing receptors – an overview. *Cell Calcium* 2004, *35*, 183–196.

Chen, G.Y., Nunez, G. Sterile inflammation, sensing and reacting to damage. *Nat. Rev.* 2010, *10,* 826-837.

Chen, J., Wu, H., Shi, Y., Li, C., Li, M. The effects of environmental salinity on trunk kidney proteome of juvenile ayu (*Plecoglossus altivelis*). *Comp. Biochem. Phys. D4* 2009, 263-267.

Chou, H.Y., Peng, T.Y., Chang, S.J., Hsu, Y.L., Wu, J.L. Effect of heavy metal stressors and salinity shock on the susceptibility of grouper (Epinephelus sp.) to infectious pancreatic necrosis virus. *Virus Res*. 1999, *63*, 121-129.

Christensen, A.K., Hiroi, J., Schult, E.T., Mc Cormick, S.D. Branchial ionocytes organization and ion transport protein expression in juvenile alewives acclimated to freshwater or seawater. *J. Exp. Biol*. 2012, *215,* 642-652.

Cuesta, A., Laiz-Carrion, R., Martin del Rio, M., Meseguer, J., Mancera, J., Esteban, M. The salinity influences humoral immune parameters of gilthead seabream (Sparus aurata). *Fish Shellfish Immun.* 2005, *18,* 255-261.

Cox, J., Neuhauser, N., Michalski, A., Scheltema, R.A., Olsen, J.V., Mann, M. Andromeda – a peptide search engine integrated into the MaxQuant environment*. J. Proteome Res*. 2011*, 10*, 1794-1805.

Cox, J., Hein, M., Luber, C.A., Paron, I., Nagaraj, N., Mann, M. Accurate Proteome-wide Label-free Quantification by Delayed Normalization and Maximal Peptide Ratio Extraction, Termed MaxLFQ. *Mol. Cell. Proteomics* 2014, *13(9*), 2513-2526.

Cutillas, P.R., Vanhaesebroeck, B. Quantitative profile of five murine core proteomes using label-free functional proteomics. *Mol. Cell. Proteomics* 2007, *6*, 1560–1573.

Das, B.C., Thapa, P., Karki, R., Das, S., Mahapatra, S., Liu, T.C., Torregroza, I., Wallace, D.P., Kambhampati, S., Van Veldhuizen, P., Verma, A., Ray, S.K., Evans, T. Retinoic acic signalling pathways in development and diseases. *Bioorg. Med. Chem*. 2014, *22*, 673-683.

Di Ciano-Oliveira, C., Thirone, A., Szászi, K., Kapus, A. Osmotic stress and the cytoskeleton, the R(h)ole of Rho GTPases. *Acta Physiol.* 2006, *187*, 257–272.

Diaz-Rosales, P., Pereiro, P., Figueras, A., Novoa, B., Dios, S. The warm temperature acclimation protein (Wap65) has an important role in the inflammatory. *Fish Shellfish Immun.* 2014, *41,* 80-92.

Eastham, J., Mpelasoka, F., Mainuddin, M., Ticehurst, C., Dyce, P., Hodgson, G., Ali, R., Kirby, M. Mekong River Basin Water Resources Assessment, Impacts of Climate Change. CSIRO, Water for a Healthy Country National Research Flagship, 2008.

Eckert, S.M., Yada, T., Shepherd, B.S., Stetson, M., Hirano, T., Grau, E.G. Hormonal control of osmoregulation in the channel catfish *Ictalurus punctatus*. *Gen. Comp. Endocr.* 2001, *122*, 270–286.

Esaki, M., Hoshijima, K., Nakamura, N., Munakata, K., Tanaka, M., Ookata, K., Asakawa K., Kawakami, K., Wang, W., Weinberg, E.S., Hirose, S. Mechanism of development of ionocytes rich in vacuolar-type H+-ATPase in the skin of zebrafish larvae. *Dev. Biol.* 2009, *329,* 166-129.

Evans, D.H., Piermarini, P.M., Choe, K.P. The multifunctional gill, dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev*. 2005, *85,* 97–177.

FAO – Food and Agriculture Organization of the United Nations. The state of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations. Rome, 2014, 243pp.

Fiol, D., Chan, S.Y., Kültz, D. Identification and pathway analysis of immediate hyperosmotic stress responsive molecular mechanisms in tilapia (*Oreochromis mossambicus*) gill. *Comp. Bioch. Physiol. D1* 2006, 244-356.

Fiol, D.F., Kültz, D. Osmotic stress sensing and signaling in fishes. *FEBS J*. 2007, *274*, 5790–5798.

Flanagan, L., Chou, J., Falet, H., Neujahr, R., Hartwig, J., Stossel, T.. Filamin A, the Arp2/3 complex, and the morphology and function of cortical actin filaments in human melanoma cells. *J. Cell Biol*. 2001*, 155*, 511–517.

Gillardin, V., Silvestre, F., Dieu, M., Delaive, E., Raes, M., Thomé , J.P., Kestemont, P. Protein expression profiling in the African clawed frog Xenopus laevis tadpoles exposed to Polychlorinated Biphenyl mixture Aroclor 1254. *Mol. Cell. Proteomics* 2009, *8*, 596-611.

Gudas, L.J. Emerging roles for retinoids in regeneration and differentiation in nomal and disease stain and diseases states. Biochimi. Biophys. *Acta* 2012, *1821*, 213-221

Gudas, L.J. Retinoids induce stem cells differentiation via epigenetic changes. *Semin. Cell. Dev. Biol*. 2013, *24,* 701-705.

Hawke, J-P., McWhorther, A.C., Steigerwalt, A.G., Brenner, D.J. *Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicaemia of catfish. *Int. J. Syst. Bacteriol*. 1981*, 31,* 396-400.

Huang, D., Sherman, B., Lempicki, R. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc*. 2009*, 4,* 44-57.

Iwata, M., Hirakiyama, A., Eshima, Y., Kagechika, H., Kato, C., Song, S.Y. Retinoic acid imprints gut homing specificity on T cells. *Immunity* 2004, *21*, 527-538.

Jiang, I-F., Khumar, V., Lee, D., Weng, C.F. Acute osmotic stress affects tilapia (*Oreochromis mossambicus*) innate immune responses. *Fish Shellfish Immun*., 2008, *25,* 41-846.

Kalujnaia, S., McWilliam, S., Zaguinaiko, V.A., Feilen, A., Nicholson, J., Hazon, N., Cutler C.P., Cramb, G. Transcriptomic approach to the study of osmoregulation in the European eel *Anguilla Anguilla***.** *Physiol. Genomics* 2007, *31,* 385-401.

Koyama, T**.**, Oike, M., Ito, Y**.** Involvement of Rho-kinase and tyrosine kinase in hypotonic stress-induced ATP release in bovine aortic endothelial cells. *J. Physiol.* 2001, *532,* 759–769.

Kültz, D., Li, J., Gardell, A., Sacchi, R. Quantitative molecular phenotyping of gill remodelling of gill in a cichlid fish responding to salinity stress. *Mol. Cell. Proteomics* 2013, *12*, 3962–3975.

Lee, H-Y., Suh, Y., Robinson, M.J., Clifford, J.I., Hong, W., Woodget, J., Cobb, M., Mangelsdorf, D., Kurie, J.M. Stress pathway activation induces phosphorylation of retinoid X receptor. *J. Biol. Chem*. 2000, *275*, 32193-32199.

Mazur, A.J., Gremm, D., Dansranjavin T, Litwin M, Jockusch BM, Wegner A, Weeds AG, Mannherz HG. Modulation of actin filament dynamics by actin binding proteins residing in lamellipodia. *Eur. J. Cell Biol*. 2010, *89,* 402-413.

Mc Cormick, S.D. Methods for nonlethal gill biopsy and measurement of NaK-ATPase activity. *Can. J. Fish. Aquatic Sci*. 1993, *50,* 656-658.

Mejillano, M.R., Kojima, S., Applewhite, D.A., Gertler, F., Svitkina, T.M., Borisy, G.G. Lamellipodial versus filopodial mode of the actin nanomachinery, Pivotal role of the filament barbed end. *Cell* 2004, *118*, 363-373.

Mora, J.R., Iwata, M., Eksteen, B., Song, S.Y., Junt, T., Otipoby, K.L., Yokota, A., Takeuchi, H., Ricciardi-Castagnoli, P., Rajewsky, K., Adams, D.H., von Andrian, U.H. Generation of gut homing Ig A secreting B cells by intestinal dendritic cells. *Science* 2006, *17,* 1157-1160.

Nath, K.A. Heme oxygenase-1, A provenance for cytoprotective pathways in the kidney and other tissues. *Kidney Int*. 2006, *70,* 432-443.

Nebel, C., Nègre-Sardagues, G., Blasco, C., Charmantier, G. Morphological ontology of the urinary system of the European sea bass *Dicentrarchus labrax. Anat. Embryol*. 2005, *209,* 193-206.

Nguyen, A.D., Savenije, H., Phan, D., Tang, D. Using salt measurements to determine the freshwater discharge distribution over the branches of a multi-channel estuary, The Mekong Delta case. *Estuar. Coast. Shelf. S.,* 2008, *77*, 423-446.

Nguyen, A.L., Dang, V.H. , Bosma, R.H. , Verreth, J.A. , Leemans, R. , De Silva, SS. Simulated impacts of climate change on current farming locations of striped catfish (*Pangasianodon hypophthalmus*, Sauvage) in the Mekong Delta, Vietnam. *Ambio,* 2014, *43*, 1509-1568.

Norton, V., Davis, K. Effect of abrupt change in the salinity of the environmental on plasma electrolytes, urine, volume, volume and electrolytic excretion in channel catfish, Ictalurus punctatus. *Comp. Bioch. Physiol.* A 1977, *56,* 425-431.

Osmanagic-Myers, S., Gregor, M., Walko, G., Burgstaller, G., Reipert, S., Wiche, G. Plectin-controlled keratin cytoarchitecture affects MAP kinases involved in cellular stress response and migration. *J. Cell Biol*. 2014, *174,* 557-566.

Papakostas, S,. Väsemagi, A., Vähä, J-P., Himberg, M., Peil, L., Primmer, C. A proteomics approach reveals divergent molecular responses to salinity in populations of European whitefish (*Coregonus lavaretus). Mol. Ecol*. 2012, *21*, 3516-3530.

Peatman, E., Baoprasertkul, P., Terhune, J., Xu, P., Nandi, S., Kucuktas, H., Li, P., Wang, S., Somridhivej, B., Dunham, R., Liu, Z. Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium. *Dev. Comp. Immunol*. 2007, *31*, 1183-96.

Peatman, E., Terhune, J., Baoprasertkul, P., Xu, P., Nandi, S., Wang, S., Somridhivej, B., Kucuktas, H., Li, P., Dunham, R., Liu, Z. Microarray analysis of gene expression in the blue catfish liver reveals early activation of the MHC class I pathway after infection with *Edwardsiella ictaluri*. *Mol. Immunol*. 2008, *45,* 553-66.

Phuc, T., Huong, D., Mather, P., Hurwood, D.A. Experimental assessment of the effects of sublethal salinities on growth performance and stress in cultured tra catfish (*Pangasianodon hypophthalmus*). *Fish Physiol. Biochem*. 2014, *40,* 1839-1848.

Poirier ,Y., Antonenkov, V., Glumoff, T., Hiltunen, J.K. Peroxisomal β-oxidation—A metabolic pathway with multiple functions. *Biochim. Biophys. Acta* 2006, *1763*, 1413-1426.

Prat, C., Bouvier, D., Comptour, A., Marceau, G., Belville, C., Clairefond, G., Blanc, P., Gallot, D., Blanchon, L., Sapin, V. All trans retinoic acid regulates aquaporins 3 expression and related cellular membrane permeability. *Placenta* 2014, *36,* 881-887.

Prodocimo ,V., Galvez, F., Freire, C.A., Wood, C.M. Unidirectional Na+ and Ca2+ fluxes in two euryhaline teleost fishes, *Fundulus heteroclitus* and *Oncorhynchus mykiss*, acutely submitted to a progressive salinity increase. *J. Comp. Physiol*. B 2007, *177*, 519–528.

Ralston-Hooper, K.J., Turner, M.E., Soderblom, E.J., Villeneuve, D., Ankley, G.T., Moseley, M.A., Hoke, R.A., Ferguson, P.L. Application of a label-free, gel-free quantitative proteomics method for ecotoxicological studies of small fish species. *Environ. Sci. Technol*. 2013, *47***,** 1091–1100.

Ratledge, C., Dover, L.G. Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.* 2000, *54*, 881-941.

Reddy,J.**,** Hashimoto, T. Peroxisomal β-oxidation and peroxisome proliferator-activated receptor α, an adaptive metabolic system. *Annu. Rev. Nutr*. 2001*, 21*, 193*–230.*

Roberts, R.J., Agius, C., Saliba, C., Bossier, P., Sung, Y.Y. Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health, a review. *J. Fish Dis*. 2010, *33*, 789-801

Rydeen, A., Voisin, N., D'Aniello, E., Ravisankar, P., Devignes, C.S., Waxman, J.S. Excessive feedback of Cyp 26A1 promotes cell non autonomous loss of retinoic acid signalling. *Dev. Biol.* 2015, *405*, 47-55.

Sha, Z., Xu, P,. Takano, T., Liu, ,J., Terhune, J., Liu, Z. The warm temperature acclimation protein Wap65 as an immune response gene, its duplicates are differentially regulated by temperature and bacterial infections. *Mol. Immunol*. 2008, *45*, 1458–1469.

Shi, Y., Chen, J., Li, C., Li, M.Y. Molecular cloning of liver Wap65 cDNA in ayu (*Plecoglossus altivelis*) and mRNA expression changes following *Listonella anguillarum* infection. *Mol. Biol. Rep*. 2010, *37*, 1523-1529.

Shigen, Y., Hua, L., Guo, Q., Zhongshi, L. First case of Edwardsiella ictaluri infection in China farmed yellow catfish *Pelteobagrus fulvidraco*. *Aquacultur*e 2009, *292*, 6-10.

Silvestre, F., Linares-Casenave, G., Doroshov, S., Kültz, D. A proteomic analysis of green and white sturgeon larvae exposed to heat stress and selenium. *Sci. Total Environ*. 2010, *408*, 3176-3188.

Tempé, D., Plechaczyk, M., Bossis, G. SUMO under stress. *Biochem. Soc. T*. 2008,*36,* 1-5.

United States Department of Agriculture (USDA) Losses caused by enteric septicaemia of catfish (ESC) 2002-09. Animal and Health Inspection Service, Veterinary Services, August 2011.

Verleih, M., Rebl, A., Köllner, B., Korytar, T., Anders, E., Wimmers, K., Goldhammer, T. Comparative molecular characterization of the regucalcin (RGN) gene in rainbow trout (*Oncorhynchus mykiss*) and maraena whitefish (*Coregonus maraena*). *Mol. Biol. Rep.* 2013, *39*, 4291-4300.

Vietnam Association of Seafood Exporters and Producers. Pangasius export statistics. VASEP, Vietnam, 2015.

Wang, Z., Boudjelal, M., Kang, S., Voorhees, J.J., Fisher, G.J. Ultraviolet irradiation of human skin caused functional vitamin A deficiency, preventable by all trans retinoic acid pre-treatment. *Nat. Med*. 1999, *5*, 418-422.

Wang, D., Zhong, X-P., Qiao, Z., Gui, J-F. Inductive transcription and protective role of fish heme oxygenase-1 under hypoxic stress. *J. Exp. Biol*. 2008, *211*, 2700-2706.

Wang, R., Fabregat, A., Rios, D., Ovelleiro, D., Foster, J.M., Cote, R.G., Griss, J., Csordas, A., Perez-Riverol, Y., Reisinger, F., Hermjakob, H., Martens, L., Vizcaino, J.A. PRIDE Inspector, a tool to visualize and validate MS proteomics data. *Nat. Biotechnol*. 2012, *30*, 135–137.

Wassman, R., Hien, N.X., Hoanh, C.T., Tuong, T.P. Sea level rise affecting the Vietnamese Mekong Delta, water elevation in the flood season and implications for Rice production. *Climatic Change,* 2004, *66*, 89-107.

Welinder, C., Ekblad, L. Coomassie staining as loading control in western blot analysis. *J. Proteome Res.* 2011, *10*, 1416-1419.

Wong, Y.F., Kopp, J.B., Roberts, C., Scambler, P.J., Abe, Y., Rankin, A.C., Dutt, N., Hendry, B.M., Xu, Q. Endogenous retinoic acid activity in principal cells and intercalated cells of mouse collecting duct system. *Plos One* 2011, *6*, 1-10.

Yada, T., Azuma, T., Takaji,Y. Stimulation of non-specific immune functions in seawater-acclimated rainbow trout *Oncorhynchus mykiss*, with reference to the role of growth hormone. *Comp. Biochem. Phys*. B 2001, *129*, 695-701.

Yamaguchi, M. Regucalcin and cell regulation, role as a suppressor protein in signal transduction. *Mol. Cell. Biochem*. 2011*, 353*, 101-137.

Yamaguchi, M. The regucalcin gene is a key in the therapy of hepatocellular carcinoma. *J. Cancer. Sci. Ther.* 2014*, 6,* 1-3.

Yuasa, K., Kholidin, E.B., Panigoro, N., Hatai, K. First isolation of Edwardsiella ictaluri from cultured striped catfish *Pangasius hypophthalmus* in Indonesia. *Fish Pathol*. 2003, *38(4)*,181-183

Chapter 6

Chronic hyperosmotic stress inhibits renal Toll-Like Receptors expression in striped catfish (*Pangasianodon hypophthalmus*, Sauvage) exposed or not to bacterial infection

Abstract

Toll-like Receptors are the first innate receptors in recognizing pathogen-associated molecular patterns. In fish, upregulation of toll-like receptors during infection has been largely demonstrated while the effects of abiotic stressors on their expression remain poorly investigated. In this study, striped catfish were submitted during 20 days to three salinity profiles (freshwater, low saline water, saline water), followed by injection of a bacterial strain of *Edwardsiella ictaluri*. The expression of TLRs 1, 3, 4, 5, 7, 9, 19, 21, 22 was measured in kidney at different time points in non infected and infected striped catfish. Infection induced overexpression of TLRs 1, 3, 4, 5, 7, 21 and 22. With elevated salinity, the expression of all TLRs, except TLR5, was severely decreased, particularly after bacterial infection. The TLRs responsiveness of striped catfish facing bacterial disease and hyperosmolarity and possible consequences on striped catfish immune response's efficiency are discussed.

Keywords: Toll-like Receptors; salinity; infection; striped catfish.

1. Introduction

Toll-like receptors (TLRs) are type I transmembrane receptors expressed by sentinel cells and specialized in recognizing specific pathogen-associated molecular patterns (PAMPs) (Kawai & Akira 2010). Innate recognition of PAMPs TLRs initiates intracellular signalling cascades that induce the production of inflammatory cytokines and inflammatory responses (Iwasaki & Medzhitov, 2004; Pasare & Medzhitov, 2004). In addition, TLRs regulate multiple mechanisms that control the initiation of the adaptive immune response. Indeed, TLRs stimulation leads to activation of co-stimulatory molecules and Major Histocompatibility Complex I/II, migration of antigen presenting cells and interaction with naive T-cells and B-cells in order to ensure a specific pathogen-driven immune response (Iwasaki & Medzhitov 2004). Mitogen-activated kinases (ERK1/2, MAPKp38), the NF-kB axis and interferon regulatory factors are key players in the signalling pathways of TLRs (West et al., 2006). In mammals, different dendritic cells subtypes and monocytes express distinct sets of TLRs on their cell surface and lysosomal compartments that might be explained by the functional specialization of sentinel cells (Iwasaki & Medzhitov 2004). Thirteen TLRs types have been identified in mammals (TLR1 to TLR13). In fish, 18 TLRs have been identified in different teleost species and they are TLR1 to TLR5, TLR7 to TLR9, TLR14, TLR16, TLR18 to TLR23, TLR25 and TLR26 (Zhang et al., 2013).

Striped catfish (*Pangasianodon hypophthalmus*, Sauvage) industry has become by far the major inland aquaculture production in Southeast Asia, particularly in the Mekong Basin in Vietnam. In 2014, striped catfish production reached 1.2 million of tons and was exported to over than 151 countries (FAO, 2014). Since 2004, several studies have pointed out the great concern regarding the effects of sea-level rise in the Mekong Basin on the agricultural development of the region (Nguyen et al., 2014). In 2016, salinity intrusion to inland reached

90 km to mouth and salinities up to 12 ppt were observed in many aquaculture provinces (SIWRR, 2016). Striped catfish does not appear to be efficient osmoregulator and seawater survival is limited due to the absence of efficient electrolyte excretion. Progressive transfer of striped catfish juveniles from freshwater to brackish water up to 20 ppt induced an elevation in plasma osmolality while gill Na^+K^+ ATPase slightly increased (Phuc et al., 2014; Schmitz et al., 2016a). In kidney proteome, 20 ppt-exposed striped catfish upregulated key proteins involved in ion transport across epithelium of ionocytes such as vacuolar-type H^+ ATPase, Na^+ K^+ ATPase and $Na^+/K^+/2Cl^-$ transporter (Schmitz et al., 2016b).

Environmental salinity is an important factor for aquatic organisms. Acclimation mechanisms from hyposmotic to hyperosmotic environment (relative to plasma) may be responsible for serious biochemical and physiological troubles such as cell shrinkage, protein decarbonylation, oxidative stress, DNA damage, cell arrest, cell and mitochondrial depolarization, promoting apoptosis (Brocker et al., 2012). In fish, hyperosmotic stress is known to simulate key factors of the innate immune system such as lysozyme and complement activity, IgM level, abundance and activity of leukocytes (Yada et al., 2000; Cuesta et al., 2005; Jiang et al., 2008; Birrer et al., 2012; Ma et al., 2014). Nonetheless, chronic hyperosmolarity may induce higher susceptibility to infectious diseases in fish (Chou et al., 1999; Schmitz et al., 2016a). In addition, several proteins involved in T cell activation and regulation were enhanced in kidney proteome of striped catfish at 10 ppt (Schmitz et al., 2017). The activation of the MAPK p38 signalling pathway, a crucial mediator in many immunological processes including inflammation and T-cells activation has been described in coho salmon, killifish and striped catfish in response to hyperosmotic stressors (Kultz & Avila, 2001; Ashwell, 2006; Maryoung et al., 2015; Schmitz et al., 2017a). Eventually, the protein TAB2, involved in B cell development and activation in response to TLR stimulation, was upregulated 4h after seawater transfer in the gill epithelium of Mozambique tilapia (Fiol et al., 2006; Ori et al., 2013). In higher vertebrates, a growing body of evidences suggests that chronic hypertonicity functions as an inflammatory mediator, triggering pro-inflammatory cytokines release, inflammatory response and proliferation of T- and B-cells (Schwartz et al., 2009; Brocker et al., 2012). The impact of hypertonicity on TLRs have been poorly investigated. A study in burn mice revealed that hypersaline infusions increased the abundance of TLR4 as well as the abundance of signalling proteins involved in the TLRs signalling transduction (i.e. NK-κB, MyD88 and MAPKp38) (Chen et al., 2010). On the contrary, TLR5 expression of broad nosed pipefish infected with *Vibrio* sp. was 3 times lower in high salinity compared to ambient salinity (Birrer et al., 2012). The present study aimed to investigate the effect of chronic hypertonicity, combined or not with an infectious disease, on the expression of distinct TLR types in striped catfish.

2. Material and methods

2.1 Fish and *in vivo* stress experiment

Investigations have been carried out in the University of Namur (Belgium) according to the guidelines for animal use and care in compliance with Belgian and European regulation on animal welfare, ethical protocol 12/189. Rationale calculations of sample size have been based on power consideration (Z_β = 0.84, α=5%). One week-old juveniles of striped catfish were imported from the Nam Sai catfish farm in Ban Sang (Thailand) and maintained in fish facilities at 28°C under constant aeration and photoperiod (12L:12D) in recirculating aquaculture systems. Fish were fed daily *ad libitum* with commercial dry pellets (Troco Supreme 4.5 mm, Coppens, The Netherlands). After 3 months, fish (40-50 g weight) were divided (30 fish/tank) into 3 experimental recirculating systems (freshwater, low saline water and saline water) each including four 100-L tanks and acclimated during 10 days. The freshwater group was

maintained in freshwater (0.4 ppt) all over the experiment. The low saline water group was exposed to a gradual water salinity increase of 0.5 ppt per day, up to 10 ppt, during 20 days. The saline water group was exposed to a gradual salinity increase of 1 ppt per day, up to 20 ppt, during 20 days. Salinity was elevated by adding marine salt (Ocean Fish, Prodac, Italy) mixed with tap water until day 20 and remained stable during the following bacterial challenge. On day 20, fish were anaesthetized in tricaine methanesulfonate (MS-222, 150 mg L^{-1}) and were intraperitoneally injected with the $LD50_{96h}$ resuspended in Hank's Balanced Salt Solution (HBSS). On days 0, 10, 20 and 23, 6 fish per tank were randomly collected with nets, immediately anaesthetized in MS-222 and euthanized by cervical dislocation. The kidney was collected by gently scratching along the vertebral column and immediately frozen in liquid nitrogen. Physicochemical data (dissolved O_2:5.7±0.5 mg L^{-1}; pH: 8.4±0.24; temperature: 28.2±0.1 °C) were measured daily in the outlet pipe using a multiparameter probe (WTW, Multi 350i). Nitrite ($N\text{-}NO_2^-$: 0.019±0.005 mg L^{-1}), nitrate ($N\text{-}NO_3^-$: 3.55±2.27 mg L-1) and ammonia ($N\text{-}NH_3^+$: 0.19±0.25 mg L^{-1}) were weekly monitored in the outlet pipe.

2.2 Bacterial challenge

Virulent strain of *Edwardsiella ictaluri* (TNA 015) was cultured at pH 7.4 and 28°C on Brain Heart Infusion (BHI) agar (Sigma-Aldrich). Colonies purity was checked after 48h according to specific shape and Gram staining in light microscopy. Bacterial counting was done in a reference bacteria solution (OD 590 nm) following Schmitz et al. (2016) by serial exponential dilution and DNA fluorochrome 4'-6'-diamidino-2-phenylindole count after filtration. The reference *E. ictaluri* solution at OD 590 nm contained approximately 10^9 CFU ml^{-1} solution. The LD50 (lethal dose 50%) 96h is10^6 CFU ml^{-1} for an inoculation volume of 0.025 ml HBSS g^{-1} fish. Infection was confirmed on the kidney by the CER group (Belgian reference laboratory for animal health, Centre d'Economie Rurale-CER, Aye, Belgium) using Biolog identification systems.

2.3 Quantitative PCR

Total RNA extraction, DNase and retrotranscription.

Isolation of RNA has been performed using Extract all® (Eurobio, Paris, France) following manufacturer's recommendation. Extraction was done with the tank as the experimental unit and kidney tissue of 6 fish were pooled together before RNA extraction. Briefly, 50 mg of kidney tissues were grinded on ice in 1 ml of ice-cold Extract all® using a bound homogenizer (Speed Mill) in RNase free tubes and precipitated in chloroform / isopropanol. The extracted RNA samples were then subjected to DNase treatment (DNase Ambion, Life Technologies) following manufacturer's recommendation to avoid DNA contamination. RNA quantification was performed by spectrophotometry using NanoDrop™ 1000 (Thermo Scientific). RNA integrity was checked by denaturating gel electrophoresis (1.2% agarose gel) as well as OD_{260} / OD_{280} and OD_{260} / OD_{230} nm absorption ratio. A starting amount of 1 µg RNA was retrotranscribed into double stranded cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) and following manufacturer's instruction. Samples were diluted 10 times for real time RT-PCR analysis.

Real time RT-PCR.

The sequences of each primer for reference genes and target genes are listed in **Table 1**. Real time PCR mastermix was prepared 3 µl nuclease free water, 1 µl primer forward (1 µM), 1 µl primer reverse (1µM) and 10 µl PowerUp™ SYBR® Green Master Mix (Life Technologies). Samples were measured in triplicates. A four step experimental run protocol was followed: denaturation (10 min at 95°C), amplification and quantification repeated 40 times (15 s at 95°C, 30 s at 60°C and 30 s at 72°C) and melting curve (40 to 95°C, heating rate 0.10°C s^{-1}) and a final cooling step (4°C). Analysis of reference and target genes was performed in triplicates.

Relative quantification of the target gene transcript was calculated following the Pfaffl method (Pfaffl 2001; Pfaffl et al., 2002), considering the Ct value and the primer efficiency. Comparison of both reference gene favours the 16S ribosome RNA primer because of its stable expression level in tested conditions and low variability. Results for gene quantification are expressed as fold expression relative to 16S rRNA. The expression level of the control in freshwater on day 0 has been designated as the value 1. Therefore, the expression ratio of tested conditions was expressed relative to the control value.

Table 1: Primers used in real-time PCR: Sequence of primers, calculated efficiency, melting temperature (Tm), targeted species and reference.

Target gene	Sequence of primer (5'-3')	Calculated efficiency	Tm (°C)	Species	Reference
TLR1	F: TCACCACGAACGAGACTTCATCC R : GACAGCACGAAGACACAGCATC	2.043	79.2	*Ictalurus punctatus*	Zhao et al., 2013
TLR3	F: GAGACTCGCTGGTTCTGATTT R: CAGAGGCCACTGGAGCAT	2.032	82.8	*Ictalurus punctatus*	Quiniou et al., (2013)
TLR4	F: TCCTTTCCAGCAACCCAATA R: CAGACCATACCTCATTGCATCA	1.999	75.3	*Ictalurus punctatus*	Quiniou et al., (2013)
TLR5	F: TGGAGGCAACGTGTTTTCG R: AAGGTGGTCAAACATGTCGAG	1.993	79.3	*Ictalurus punctatus*	Quiniou et al., (2013)
TLR7	F: CTGTCCATCTCAAGCCATCTC R : GCCGTGTCAGTTCTATCGTAG	1.970	81.9	*Ictalurus punctatus*	Zhao et al., 2013
TLR9	F: TAGCCTTAGACCTCTGTTCAAC R : CACAACCATTCTCAACGATCTC	1.997	77.2	*Ictalurus punctatus*	Zhao et al., 2013
TLR19	F: CACTCACTGGAACTGTTGTATC R : ACCTGTGCTCGTGTATTCTG	1.967	81.3	*Ictalurus punctatus*	Zhao et al., 2013
TLR21	F: GAGCAGTGGCGTCGTCTTC R : CGGTGGTGGAGGCAAAGTC	1.988	82.4	*Ictalurus punctatus*	Zhao et al., 2013
TLR22	F: CCTTCTGGTGTCGTTCATTTATC R : TATCCGTGTTGCTGGTGTATC	1.991	79.2	*Ictalurus punctatus*	Zhao et al., 2013
Reference genes					
ß-actin	F : TGTATCGCCTCTGGTCGT R : AAGCTGTAGCCTCTCTCG	1.872	83.1	*Pangasianodon hypophthalmus*	Sinha et al., (2010)
16S rRNA	F: TATCTTCGGTTGGGGCG R : CCTGATCCAACATCGAGG	1.970	80.8	*Pangasianodon hypophthalmus*	Sinha et al., (2010)

2.4 Statistical analysis

Heterogeneity of variances was tested by Levene test and normality was checked by Shapiro-Wilk test. Significant changes were analysed by three-ways analysis of variance ANOVA 3 (3 factors: "salinity", "time", "infection"), followed with pairwise multiple comparisons procedures by Scheffe test ($p<0.05$) in SigmaStat. Data are represented as the mean ± SEM and tanks were used as the statistical unit ($n = 4$, pool of 6 fish).

3. Results and discussion

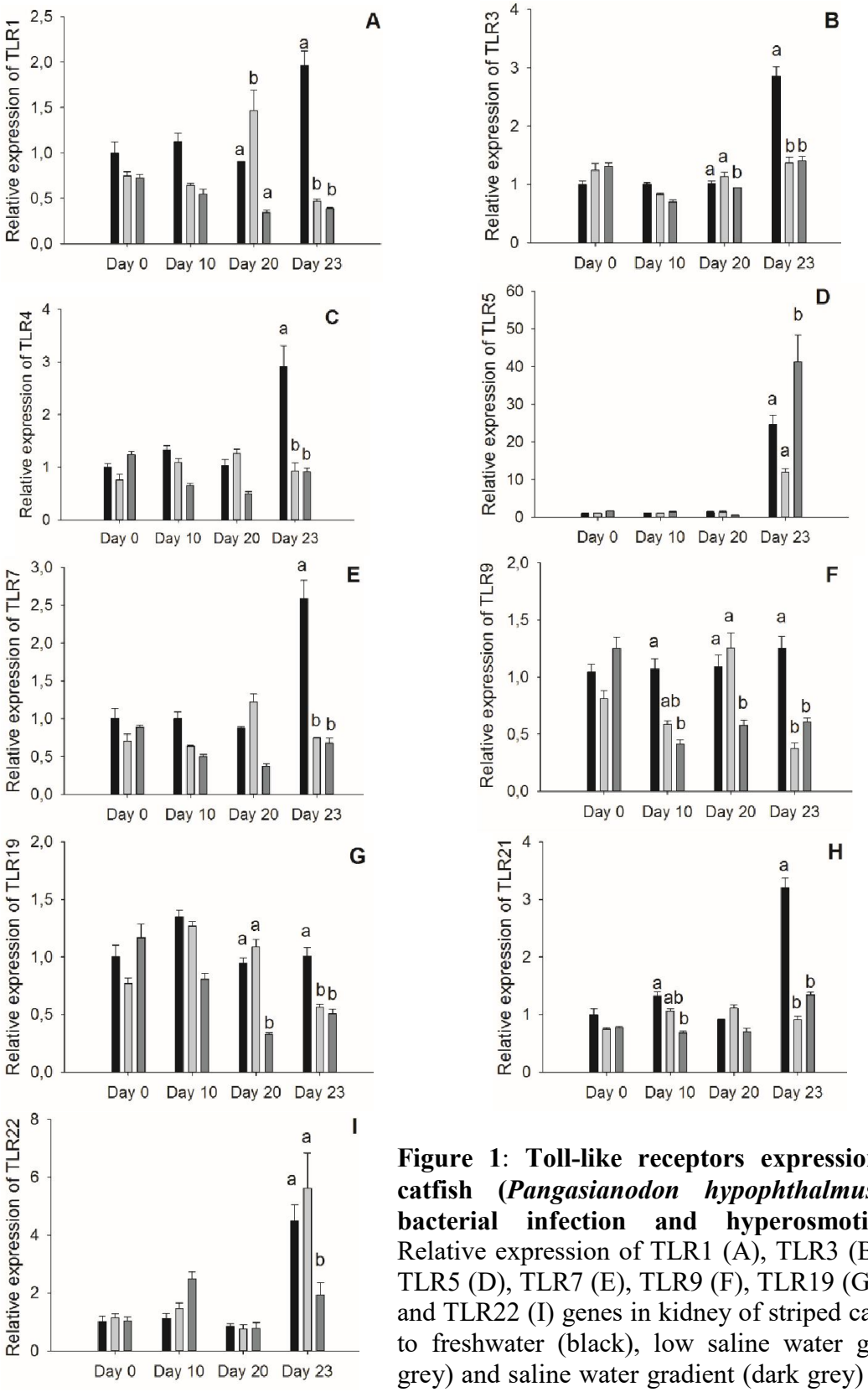


Figure 1: **Toll-like receptors expression in striped catfish (*Pangasianodon hypophthalmus*) following bacterial infection and hyperosmotic stressors.** Relative expression of TLR1 (A), TLR3 (B), TLR4 (C), TLR5 (D), TLR7 (E), TLR9 (F), TLR19 (G), TLR21 (H) and TLR22 (I) genes in kidney of striped catfish exposed to freshwater (black), low saline water gradient (light grey) and saline water gradient (dark grey) on day 0, 10, 20 and 23 (3 days after infection). The values were presented as the mean ± SD with n=4 (4 tanks, 6 fish/tank).The statistical letters (a,b) indicate a significant change ($p<0.05$) between salinity treatments on the same sampling day.

In this study, we investigated the expression profiles of TLRs 1, 3, 4, 5, 7, 9, 19, 21 and 22 in kidney of striped catfish during chronic hyperosmotic stressors up to 10 and 20 ppt, before and 3 days after injection of a virulent strain of the intracellular bacteria *Edwardsiella ictaluri*.

Our results indicates that infection significantly increased the expression of TLRs 1 (2.0 fold), 3 (2.7 fold), 4 (2.9 fold), 5 (24.0 fold), 7.0 (2.6 fold), 21 (3.2 fold) and 22 (4.5 fold) of fish raised in freshwater ($p<0.001$) but not the expression of TLRs 9 and 19 (Figure 1). In fish, TLR signalling pathways exhibit particular characteristics distinct from that of mammals and the ligand-bind properties for distinct TLR remain poorly evaluated. To date, upregulation of several TLRs subtypes in fish upon bacterial infection has been largely demonstrated. Hence, hybrid catfish submitted to *E. ictaluri* displayed an increased expression of TLR3 and TLR5 in kidney to reach, 5 days post-infection, values respectively 25 and 5 fold higher than those of non-exposed fish (Bilodeau et al., 2006). In addition, channel catfish overexpressed TLR1 (4.4 fold), TLR3 (2.5 fold), TLR4 (2.9 fold), TLR21 (4.3 fold) and TLR22 (1.75 fold) (3.0 fold) in kidney upon infection with the protozoan *Ichthyophthirius multifiliis*, as well as TLRs 3, 9, 19 and 25 in other organs, including skin and gills, the primary sites of infection of *I. multifiliis* (Zhao et al., 2013). In other fish species, TLRs 2, 3, 5, 9, 21 and 22 have been shown to participate in the recognition of specific bacterial ligands while TLRs 1, 4, 14, 18, 25 may sense bacterial PAMPs (Zhang et al., 2014).

Regarding the impact of salinity on Toll-like receptors expression, salinity slightly decreased TLRs expression before infection, especially TLRs 9 and 19. However, at the exception of TLR5, salinity strongly inhibited the increase of TLRs expression during infection. In mammals, hyperosmotic stressor activates the MAPK14 signalling pathways and triggers pro-inflammatory cytokines release, activation of neutrophils and proliferation of T-cells (Brocker et al., 2012; Schwartz et al., 2009). Similarly, our previous work showed that in healthy and infected fish, hyperosmotic stressors enhanced several inflammatory functions (e.g. respiratory burst, lysozyme activity, phagocytosis, number of circulating leukocytes as well as abundance of inflammatory mediators, members of the MAPK14 signalling pathway, T-cells regulators and activators in kidney) (Schmitz et al., 2016; Schmitz et al., 2017a). However, these apparent immunostimulatory effects were accompanied by higher mortalities, especially in saline water (20 ppt) condition (Schmitz et al., 2016). Therefore, it is likely that the sterile inflammation response induced by the hyperosmotic condition inhibits the engagement of TLRs on key adaptive functions including activation of pathogen-specific immune response and lead to lower efficient immune responses and eventually higher susceptibility to bacterial disease.

Particularly, TLR5 may increase more than 40 fold during infection, which is 10 fold higher than other TLRs. In addition, at the opposite of other TLRs, expression was stimulated in saline water compared to freshwater and low saline water. In mammals, TLR5 recognizes bacterial flagellin, which contributes to motility and has the particularity of being expressed on the cell surface of non-immune cells including epithelial cells (Hayashi et al., 2001; Vijay-Kumar et al., 2008). In addition, in fish, the presence of soluble TLR5 has been described in puffer fish (*Takifugu rubripes*), Japanese flounder (*Paralichthys olivaceus*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) and channel catfish (*Ictalurus punctatus*) (Oshiumi et al., 2003; Tsujita et al., 2004; Baoprasertkul et al., 2007; Hwang et al., 2010). The expression of these TLR5s paralogs plays important role in the immune response against bacterial microbes and has the capacity to amplify the danger in a positive feed loop which might explained the relatively higher expression rate of TLR5 compared to other TLRs (Rebl et al., 2009; Hwang et al., 2010).

However, as TLRs expression is restricted to sentinel cells such as dendritic cells and macrophages, TLRs expression fold changes relative to ubiquitous housekeeping genes might at least partly depend on sentinel cells abundance and localization and not only on the expression rate. For instance, upon infection, sentinel cells might migrate up to

melanomacrophage centers in head kidney for antigen presentation and hence indirectly locally increase the relative abundance of TLRs transcripts. Similarly, salinity might impair migration of sentinel cells to kidney and hence indirectly decrease the abundance of TLRs transcripts in the kidney. In mammals, salinity is known to affect the activation of specific immune mecanisms. Hypertonic stressors impair leukocytes' adhesion molecules function and inhibit cell endothelial activation upon infection, thereby might unbalance leukocytes motility and distribution (Murao et al., 2000; Junger et al., 2012). Then, hyperosmotic media inhibits *in vitro* receptor-mediated endocytosis in fibroblast by blocking the formation of clathrin-coated pit (Heuser & Anderson, 1989). Furthermore, hypertonic loading of antigens reduced antigen-procressing speed and increased the risk of pinosomes lysis, cytoplasmic delivery of antigen and hence antigen-presentation to MHCI followed by cytotoxic T-cells induction (Enders, 2002). Eventually, the head kidney assumes the haematopoietic function in fish in the absence of bone marrow. Sothe abundance of TLRs transcripts may be biased by variation in cells proliferation ratio in response to salinity and / or infection. In the future, it might be interesting to complement these results with *in vitro* experiments or to correlate TLRs expression changes with abundance profiles of specific immune cells types.

4. Conclusions

In kidney of striped catfish, bacterial infection with *Edwardsiella ictaluri* increased the abundance of TLRs transcripts in kidney whereas salinity decreased it, particularly during infection. Our previous work showed that such hyperosmotic stressors stimulated several innate immune factors such as inflammation response and proliferation of T-cells markers but fish were suffering from higher susceptibility to disease. Therefore, it is likely that hyperosmotic stressors depresses the activation of TLRs-induced genes, therefore preventing efficient pathogen-specific response through T-cells activation by antigen-presenting cells and eventually leading to less efficient immune response. However, whether expression changes in TLRs are due to a direct effect of infection/salinity on the expression rate or of an indirect effect due to changes in sentinel cell migration remain to be investigated.

Acknowledgements: This work was supported by the Fonds de la Recherche dans l'Industrie et l'Agriculture (Wallonia-Brussels Federation) (grant number) number 14768837, providing a grant to MS. Special thanks are also due to BELSPO for their support within the framework of the IAP project Aquastress (P7/31).

References

Ashwell, J.D. The many paths to p38 mitogen-activated protein kinase activation in the immune system. Nat. Rev. Immunol. 2006, *6*, 532-540.

Baoprasertkul, P., Xu, P., Peatman, E., Kucuktas, H., Liu, Z. Divergent Toll-like receptors in catfish (Ictalurus punctatus): TLR5S, TLR20, TLR21. *Fish Shellfish Immunol.*, 2007, *23*(6), 1218-1230.

Bilodeau, A. L., Peterson, B. C., Bosworth, B. G. Response of toll-like receptors, lysozyme, and IGF-I in back-cross hybrid (F1 male (blue× channel)× female channel) catfish challenged with virulent Edwardsiella ictaluri. *Fish Shellfish Immunol*, 2006, *20*(1), 29-39.

Birrer, S.C., Reusch, T.B.H., Roth, O. Salinity changes impair pipefish immune defence. *Fish Shellfish Immunol*. 2012, *33,* 1238-1248.

Brocker, C., Thompson, D., Vasiliou, V. The role of hyperosmotic stress in immunity and disease. *Biomol. Concepts* 2012, *3(4)*, 345-364.

Chen, L-W., Su, M-T., Chen, P-H., Liu, W-C., Hsu, C-M. Hypertonic saline enhances host defense and reduces apoptosis in burn mice by increasing Toll-like receptors. *Shock,* 2010, *35(1),* 59-66.

Chou, H.Y., Peng, T.Y., Chang, S.J., Hsu, Y.L., Wu, J.L. Effect of heavy metal stressors and salinity shock on the susceptibility of grouper (*Epinephelus* sp.) to infectious pancreatic necrosis virus. *Virus Res.* 1999, *63,* 121-129.

Cuesta, A., Laiz-Carrion, R., Martin del Rio, M., Meseguer, J., Mancera, J., Esteban, M. The salinity influences humoral immune parameters of gilthead seabream (*Sparus aurata*). *Fish Shellfish Immun.,* 2005, *18*, 255-261.

Enders, G. A. Mechanism of antigen presentation after hypertonic loading of soluble antigens. *Immunology*, 2002, *106*(4), 511-516.

Jiang, I-F., Khumar, V.B., Lee, D.N., Weng, C.F. Acute osmotic stress affects tilapia (*Oreochromis mossambicus*) innate immune responses. *Fish Shellfish Immun*. 2008, *25,* 41-846.

FAO – Food and Agriculture Organization of the United Nation. The state of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations. Rome, 2014, pp. 243.

Fiol, D., Chan, S.Y., Kültz, D. Identification and pathway analysis of immediate hyperosmotic stress responsive molecular mechanisms in tilapia (*Oreochromis mossambicus*) gill. *Comp. Bioch. Physiol. D1* 2006, 244-356.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T., Yi, E.C. , Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D., Aderem, A. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature,* 2001, *410*, 1099–1103

Heuser, J. E., Anderson, R. G. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *The Journal of cell biology*, 1989, *108*(2), 389-400.

Hwang, S. D., Asahi, T., Kondo, H., Hirono, I., Aoki, T. Molecular cloning and expression study on Toll-like receptor 5 paralogs in Japanese flounder, Paralichthys olivaceus. *Fish Shellfish Immunol.*, 2010, *29*(4), 630-638.

Iwasaki,A., Medzhitov, R. Toll-like receptors control of the adaptive immune responses. *Nat. Immunol*., 2004, *5(10*), 987-995.

Junger, W., Rhind, S.G. , Rizoli, S.B., Cuschieri, J., Shiu, M.Y., Baker, A.J., Li, L., Shek, P.N., Hoyt, D.B., Bulger, E.M. Resuscitation of traumatic hemorrhagic shock patients with hypertonic saline - without dextran - inhibits neutrophil and endothelial cell activation. *Shock*, 2012, *38(4),* 341-350.

Kawai, T., Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol*., 2010, 11, 373–384.

Kültz, D., Avila K. Mitogen-activated protein kinases are in vivo transducers of osmosensory signals in fish gill cells. *Comp. Biochem. Physiol. B,* 2011, *129*, 821-829.

Ma, Z., Zheng, P., Guo, H., Jiang, S., Qin, J.G., Zhang, D., Liu, X. Salinity regulates oxidant enzyme and Na^+K^+-ATPase activities of juveniles golden pomprano *Trachinotus ovatusi* (Linnaeus 1758). *Aquaculture Res*., 2014, *47*(5), 1481-1487.

Maryoung, L.A., Lavado, R., Bammler, T., Gallagher, E., Stapleton, P., Beyer, R., Farin, F., Hardiman, G., Schlenk, D. Differential Gene Expression in Liver, Gill, and Olfactory Rosettes of Coho Salmon (*Oncorhynchus kisutch*) After Acclimation to Salinity. *Mar. Biotechnol.* 2015, *17(6),* 703-717.

Murao, Y., Hoyt, D. B., Loomis, W., Namiki, S., Patel, N., Wolf, P., Junger, W. G. (2000). Does the timing of hypertonic saline resuscitation affect its potential to prevent lung damage ? *Shock, 2000, 14*(1) 18-23.

Nguyen, A.L., Dang, V.H., Bosma, R.H. , Verreth, J.A. , Leemans, R. , De Silva, SS. Simulated impacts of climate change on current farming locations of striped catfish

(*Pangasianodon hypophthalmus*; Sauvage) in the Mekong Delta, Vietnam. *Ambio, 2014*, *43*, 1509-1568.

Ori, D., Kato, H., Sanjo, H., Tartey, S., Mino, T., Akira, S., Takeuchi, O. Essential roles of K63-linked polyubiquitin-binding proteins TAB2 and TAB3 in B cell activation via MAPKs. *J. Immunol*. 2013, *190(8*), 4037-45.

Pasare, C., Medzhitov, R. Toll-like receptors: linking innate and adaptive immunity. *Microbes Infect*., 2004, *6*, 1382-1387.

Phuc, T.H.N., Huong, D.T.T., Mather, P.B., Hurwood, D.A. Experimental assessment of the effects of sublethal salinities on growth performance and stress in cultured tra catfish (*Pangasianodon hypophthalmus*). *Fish Physiol. Biochem*., 2014, *40,* 1839-1848.

Quiniou, S.M.A., Boudinot, P., Bengten, E. Comprehensive survey and genomic characterization of Toll-Like Receptors (TLRs) in channel catfish, *Ictalurus punctatus*: Identification of novel fish TLRs. *Immunogenetics*, 2013, *65,* 511-530.

Rebl, A., Goldammer, T., Seyfert, H. M. Toll-like receptor signalling in bony fish. *Vet. Immunol. Immunopathol.*, 2010, *134*(3), 139-150.

Schmitz, M., Douxfils, J., Mandiki, S., Morana, C., Baekelandt, S., Kestemont, P. Chronic hyperosmotic stress interferes with immune homeostasis in striped catfish (*Pangasianodon hypophthalmus*, S.) and leads to excessive inflammatory response during bacterial infection. *Fish Shellfish Immunol.*, 2016a, *55,*550-558.

Schmitz, M., Mandiki, S., Douxfils, J., Ziv, T., Admon, A., Kestemont, P. Synergic stress in striped catfish (*Pangasianodon hypophthalmus*, S.) exposed to chronic salinity and bacterial infection: effects on kidney protein expression profile. *J. Proteomics,* 2016b, *142,* 91-101.

Schmitz, M., Mandiki, S.N.M., Ziv, T., Admon, A., Kestemont, P. Low saline stress enhanced physiological and immune pathways in striped catfish *Pangasianodon hypophthalmus* (Sauvage). *Proteomics,* 2017 (in review)

Schwartz, L., Guais, A., Pooya, M., Abolhassani, M. Is inflammation a consequence of extracellular hyperosmolarity? *J. Inflamm*., 2009, *6*(21).

Sinha, A.K., Vanparys, C., De Boeck, G., Kestemont, P., Wang, N., Phuong, N.T., Scippo, M-L., De Coen, W., Robbens, Y. Expression characteristics of potential biomarkers genes in tra catfish, *Pangasianodon hypophthalmus*, exposed to trichlorofon. *Comp. Biochem. Physiol. D,* 2010, *5,* 207-216.

SIWRR-The Southern Institute of Water Resources Research (2016) Saline intrusion in the Mekong Delta. http://www.tongcucthuyloi.gov.vn/, *accessed on March 24, 2016.*

Tsujita, T., Tsukada, H., Nakao, M., Oshiumi, H.,Matsumoto, M., Seya, T. Sensing bacterial flagellin by membrane and soluble orthologs of Toll-like receptor 5 in rainbow trout (*Onchorhynchus mikiss*). *J. Biol. Chem*., 2004, *279,* 48588–48597.

Vijay-Kumar, M., Aitken, J.D., Gewirtz, A.T. Toll-like receptor-5: protecting the gut from enteric microbes. *Semin. Immunopathol.*, 2008, *30*, 11–21.

West, A.P., Koblansky, A.A., Ghosh, S. Recognition and signaling by toll-like receptors. *Annu. Rev. Cell Dev. Biol.*, 2006, 22, 409–437.

Yada, T., Azuma, T., Takaji, Y. Stimulation of non-specific immune functions in seawater-acclimated rainbow trout *Oncorhynchus mykiss*, with reference to the role of growth hormone. *Comp. Biochem. Phys. B*, 2001, *129*, 695-701.

Zhang, J., Liu, S., Rajendran, K.V., Sun, L., Zhang, Y., Kucuktas, H., Liu, H., Liu, Z. Pathogen recognition receptors in channel catfish: III Phylogeny and expression analysis of Toll-like receptors. *Dev. Comp. Immunol*., 2013, *40(2*), 185-194.

Zhang, J., Kong, X., Zhou, C., Li, L., Nie, G., Li, X. Toll-like receptor recognition of bacteria in fish: ligand specificity and signal pathways. *Fish Shellfish Immunol*, 2014, *41*(2), 380-388.

Zhao, F., Li, Y-W., Pan, H-J., Shi, C-B., Luo, X-C., Li, A-X., Wu, S-K. Expression profiles of toll-like receptors in channel catfish (*Ictalurus punctatus*) after infection with *Ichthyophthirius multifiliis*. *Fish Shellfish Immunol.,* 2013, *35,* 993-997.

Chapter 7

General discussion

1. Overview of the thesis

Food fish production plays a crucial role in human food security and nutritional needs worldwide. Owing to the rapid population growth rate and the declining fisheries, politics and governments have turned to the aquaculture sector (FAO, 2016). However, the sustainability of fisheries and aquaculture development is currently suffering from increasing pressure of climate change and human activities. Overfishing, land-use changes, sea level rise, damming, acidification, extreme events, changes in rainfall and surface temperature are challenging worldwide aquaculture and fisheries resources, inducing both negative and positive effects on the sustainability of these sectors. These changes occur for example through modification in fish growth rate, food conversion rate or immune changes (FAO, 2016). In this thesis, we focused our attention on the impact of salt intrusions, mainly induced by sea level rise and upstream damming, into the Mekong Delta, a region with considerable aquaculture production. In the Mekong catchment, salinity increase was correlated with reducing growth rates, higher mortality rates and lower yield in the striped catfish sector and threatens striped catfish sector's perenity (Phan et al., 2009; De Silva & Phuong 2011; Kam et al., 2012). The main objective of this work was to better understand the mechanisms involved in the effects of hyperosmolarity on the immune system of the striped catfish (*Pangasianodon hypophthalmus*) and on its susceptibility to disease.

In chapter 1, an introductive review summed up the consequences of hyperosmotic stressors on the animal immune system and the initiation and progression of diseases, in relation with the evolution of innate and adaptive immune defences. We observed that the consequences of hyperosmolarity on the immune defences mainly depend on osmotic stressor's intensity, exposure time (acute versus chronic) and the acclimation capacity of the organism. In invertebrates (i.e. crustaceans and mollusks), acute stressors result in immunodepression and lower resistance to bacterial and viral diseases. In vertebrates (i.e. fish and mammals), acute and chronic exposure triggers inflammatory responses and positively modulates the immune response, especially the innate immunity. Prolonged exposure to hyperosmotic stressors has been related to excessive inflammatory responses, tissue damage and chronic inflammatory diseases. The existence of "osmotic surveillance" mechanisms in vertebrates, evolved with body compartmentalization and tightly linked to immune defences, might be at least partly responsible for intensification of inflammatory and immune responses. Moreover, we highlighted important differences in resistance to abiotic stress among species, meaning that species living in fluctuating habitats were more resistant to saline stress compared to species living in more stable habitats (Lenz et al., 2011; Mattozzo et al., 2012). Eventually, we pointed out the importance of the Rel/NF-kB/NFAT family, leading to activation of the MAPK p38 (or MAPK14) cascade in linking osmoregulatory and immune responses.

In chapter 2, we performed a field study in order to investigate the hypothetic impacts of seasonal chronic hyperosmotic stressor (mainly runoff and salinity fluctuations) in striped catfish under aquaculture conditions. In tidal sites, plasma osmolality, abundance of immune cells, lysozyme activity increased compared to non-tidal sites whereas kidney complement activity decreased. Moreover, plasma osmolality, gill NaK ATPase increased during the dry season whereas plasma lysozyme activity and kidney complement activity decreased. However, the work does not provide regular data neither on physicochemical analysis (e.g. oxygen,

suspended solids, pollutants) nor on the microorganisms content. Therefore, results interpretation is strongly hampered with confounding factors.

In the following chapters (chapters 3, 4, 5 and 6), we isolated the effects of three salinity profiles (freshwater, 10 ppt and 20 ppt) during 20 days on the immune response of striped catfish, followed by infection with a virulent bacteria, *Edwardsiella ictaluri,* responsible for the enteric septicemia of catfish. With elevated salinity, plasma osmolality severely increased while gill Na^+K^+ATPase slightly increased. Salinity alone stimulated the number of granulocytes, lysozyme activity and respiratory burst but depleted the number of thrombocytes. Salinity in combination with infection stimulated the number of monocytes and ACH50. On the contrary, erythrocytes, hematocrit, heat shock protein 70 and high mobility group box 1 did not significantly vary with salinity profiles. Then, salinity induced an earlier onset of mortalities after *E. ictaluri* inoculation whereas cumulative mortality reached 79.2%, 67.0% and 91.7% respectively in freshwater, low saline water and saline water. In the kidney, in the low saline gradient, hierarchical clustering showed that the effect of salinity on protein abundance is masked by the infection status. On the contrary, in the higher saline gradient, the abundance of proteins is driven by the synergy of both salinity and infection. Saline stressor was responsible for upregulation of ion transporters as well as proteins involved in the synthesis of osmotically active metabolites (i.e. aminoacids and derivatives, polyamines, carbohydrates, polyols and urea). Moreover, infection and salinity upregulated chaperones as well as various proteins related to cytoskeleton network organization and focal adhesion assemblage. Regarding the published literature and our results, we hypothesized that salinity and infection induced a migration of actin filament and a reorganization of an actin cytoskeleton to the periphery. At 20 ppt, our results suggested a collapse of the extracellular matrix, intermediate filament network and spectrin scaffold. Furthermore, we showed that salinity upregulated antioxidant defences and cytochrome P450 1A, involved in detoxification process. Specifically, in the low salinity profile, proteins involved in T cells regulation, phagocytosis and MAPK p38 signalling cascade were upregulated with salinity and infection. In the higher salinity profile, iron-binding proteins and complement proteins were downregulated with salinity whereas infection upregulated these proteins. Eventually, proteins involved in the metabolism of retinoic acid were downregulated with salinity and infection.

Although scientific literature revealed that, in mammals, salinity stimulates lymphopoiesis and T-cells immune response, and that our proteomics results were consistent with this hypothesis, we wonder how salinity may affect the process of antigen-presentation and the subsequent activation of T-cells specific immune response. Therefore, we complemented this work with a brief analysis of the expression of Toll-like receptors in the kidney. We observed that salinity downregulated the expression of all membranous Toll-like receptors (but did not affect the expression of soluble Toll-like receptor 5). Particularly, we observed that overexpression of Toll-like receptors in response to bacterial infection was inhibited by the saline stressor.

2. Acute and chronic stressors modulate the immune defences: mechanisms of cross tolerance and synergy

Stressor may induce suppressive or enhancing immune responses, the outcome will depend on the intensity and duration of the stressor (Tort, 2011). Previous exposure to stress will determine the ability of an organism to build a further response and therefore the efficiency of other systems such as the immune system. However, cellular and molecular mechanisms were not fully understood and our current knownledge does not allow the prediction of the consequences of a particular stressor on the immune defences (Tort, 2011). Under certain conditions, acute/short-term stressors may be stimulatory and immune responses show an

activating phase, especially regarding innate defences (Tort, 2011). Acute handling stress in dab *Limanda limanda* induced leukocytes mobilization (especially phagocytes) and enhanced respiratory burst activity (e.g. Pulsford et al., 1994). In Mozambique tilapia (*Oreochromis mossambicus*) and Nile tilapia (*Oreochromis niloticus*), acute osmotic shocks induced respiratory burst, complement and lysozyme activity (Jiang et al., 2008; Choi et al., 2013). At the opposite, chronic/long-term stressors rather lead to immunodepression and the chances for infection may be enhanced (Tort, 2011). For instance, epidemiological studies have linked a variety of stressors (social stressor, nutritional change, primary viral infection) with an increased incidence and severity of respiratory infections in mammals (Hodgson et al., 2005). In fish, chronic stresses such as chronic confinement or crowding result in a chronic elevation of plasma cortisol, decrease in immune defences and increased mortality due to common microbial infections (e.g. Pickering & Pottinger, 1989).

In multi-stress approaches, the effects of stressors may be synergic or further induced cross tolerance mechanisms. For instance, DuBeau et al. (1998) reported that a slight thermal shock (15 min at 26°C) in Atlantic salmon *Salmo salar* induces upregulation of heat shock protein 90 and higher tolerance to osmotic shock (45 ppt). Another example is the so-called "endotoxin or LPS tolerance", defined as the lower immune capacity of cultured macrophages (in vitro) or the host (in vivo) to respond to LPS activation following a first exposure to this stimulus (Fan et al., 2004). It has been shown that induced bacterial lipoprotein tolerance may protect from lethal microbial shock by live bacteria and endotoxic shock (Wang et al., 2003). The downregulation of TLR2 and 4 signalling cascades, which involves downstream molecules such as MyD88 and MAPK14 for NF-kappaB activation has been pointed out in cross tolerance mechanisms in fish and mammals (Li et al., 2006; Novoa et al., 2009). On the contrary, the effect of stressors might be additional, leading to the intensification of the stressor effects with risk of sepsis during infectious diseases. In mammals, primary exposure to herpes virus-1 and maternal separation increased innate immune responses that correlates with acute inflammatory responses and higher mortality following a secondary bacterial respiratory infection (Hodgson et al., 2005). Similarly, in this study, hyperosmotic stress and infection act in synergy to modulate protein's abundance in kidney as well as investigated immune factors.

3. What's link between osmoregulation and immunity? Discussion and hypothesis.

The effects of chronic hyperosmotic stressor on the cellular and immune defences of striped catfish observed in this study are summarized in **Figure 1.** In fish, tolerance to salinity may greatly depend on the acclimation capacity of the species (euryhaline/stenohaline). Striped catfish does not appear to be an efficient osmoregulator and survival in brackish water is limited due to the absence of efficient electrolyte excretion. Progressive transfer of striped catfish juveniles from freshwater to brackish water induces an elevation in plasma osmolality. These changes are accompanied by a slight increase in gill Na^+K^+ ATPase, suggesting a weak capacity to regulate the osmotic pressure and ion content. In kidney proteome, we further observed that salt-stressed striped catfish upregulated ion transporters, suggesting that striped catfish, such as channel catfish, may adapt its renal salt excretory capacity (Norton & Davis, 1977; Eckert et al., 2001). Furthermore, upregulation of proteins involved in the biosynthesis of organic osmolytes (e.g. taurine, proline, spermidine) may increase the intracellular osmotic pressure, therefore preventing excessive turgescence and cell shrinkage.

In verterates, local osmotic gradients serve as signal to monitor the tissue barrier integrity. Therefore, disruption of epithelial osmotic homeostasis is perceived as "danger signal", trigerring the inflammatory response and the activation of innate immune defences. This process has been largely described in mammals and in a lesser extent in zebrafish (Srinivas,

2012; Enyedi et al., 2013). For example, in human cornea, osmotic stress stimulates osmosensors and induces the production of inflammatory cytokines (particularly TNF-α) by immune cells which mobilize the activity of Rho family small GTPases and activate the MAPK p38 cascade, leading to microtubule disassembly, actin remodelling and cortical actin contraction (Srinivas, 2012). Finally, contraction of cortical actin cytoskeleton breaks down junctional complexes, leading to loss in barrier integrity, increase in permeability thereby further accelerating the inflammatory response (Srinivas, 2012). In our study, salinity upregulated several proteins involved in the MAPK p38 cascade and in the reorganization of actin cytoskeleton while lowered the abundance of spectrin scaffold, intermediate filaments and extracellular matrix components. Therefore, it might be possible that similar processes than those described in mammals and zebrafish occurred in this study.

Throughout evolution, proteins of the Rel/NF-kB/NFAT family and consequent activation of MAPK p38 cascade had critical roles in linking responses to osmoregulation and immunity. In mammals, this family of proteins is known to regulate the expression of genes involved in osmoprotection (e.g. ion transporters, osmolyte biosynthesis, chaperones) (Neuhofer et al., 2010). In addition, their upregulation in mammals during hyperosmotic stress has also been correlated to production of inflammatory cytokines, activation of innate immune cells and proliferation and activation of T- and B-cells (Neuhofer et al., 2010; Buxadé et al., 2012). In vertebrates, it has been largely demonstrated *in vitro* and *in vivo* that chronic and acute hyperosmotic stressors lead to secretion of pro-inflammatory cytokines, proliferation and activation of immune cells (cfr **Table 1** in **Chapter 1** for references). In addition, many chronic inflammatory disorders such as colonic inflammation, diabetes and eyes chronic disorders are related to hyperosmolarity. In this study, we showed that 20-days exposure to hyperosmotic stressors induced proliferation of innate immune cells and stimulation of innate parameters such as lysozyme activity, respiratory burst, phagocytosis and complement. Moreover, in this study, several proteins thought to be involved in T cell regulation and lymphoid cell development were upregulated in kidney during moderate saline stress. Nonetheless, poor information is available regarding the effect of salinity on the specific response. In this study, it has been demonstrated that both saline profiles lowered the abundance of Toll-like receptors transcripts in the kidney, especially in infected fish. Whether this decrease is induced by a problem of migration of sentinel cells or a downregulation of Toll-like receptors through a negative feedback loop following chronic activation of sentinel cells is unknown and has never been investigated yet. In fish, T-cells are activated in the kidney and required the presence of inflammatory cytokines but also the interaction with mature sentinel cells. Therefore, it is plausible that chronic salinity prevents the activation of adaptive cells and the development of an efficient specific response during pathogen attack.

In conclusion, on one hand, stimulation of MAPK p38 by osmosensors and the presence of an invader may lead to exagerating inflammatory response. Prolonged activation of unspecific mechanisms such as production of radicals and proteolytics enzymes might become deleterious for the fish, leading to tissue destruction, decrease in the fish health status and thereby may favour bacterial replication. On the other hand, prevention of T-cells differentiation / activation prevents the fish to develop an efficient specific response in order to bridge the inability of the innate immunity to limit the propagation of the invader. This hypothesis may explain the general decrease in the health status of striped catfish, associated with earlier and higher mortalities during bacterial disease.

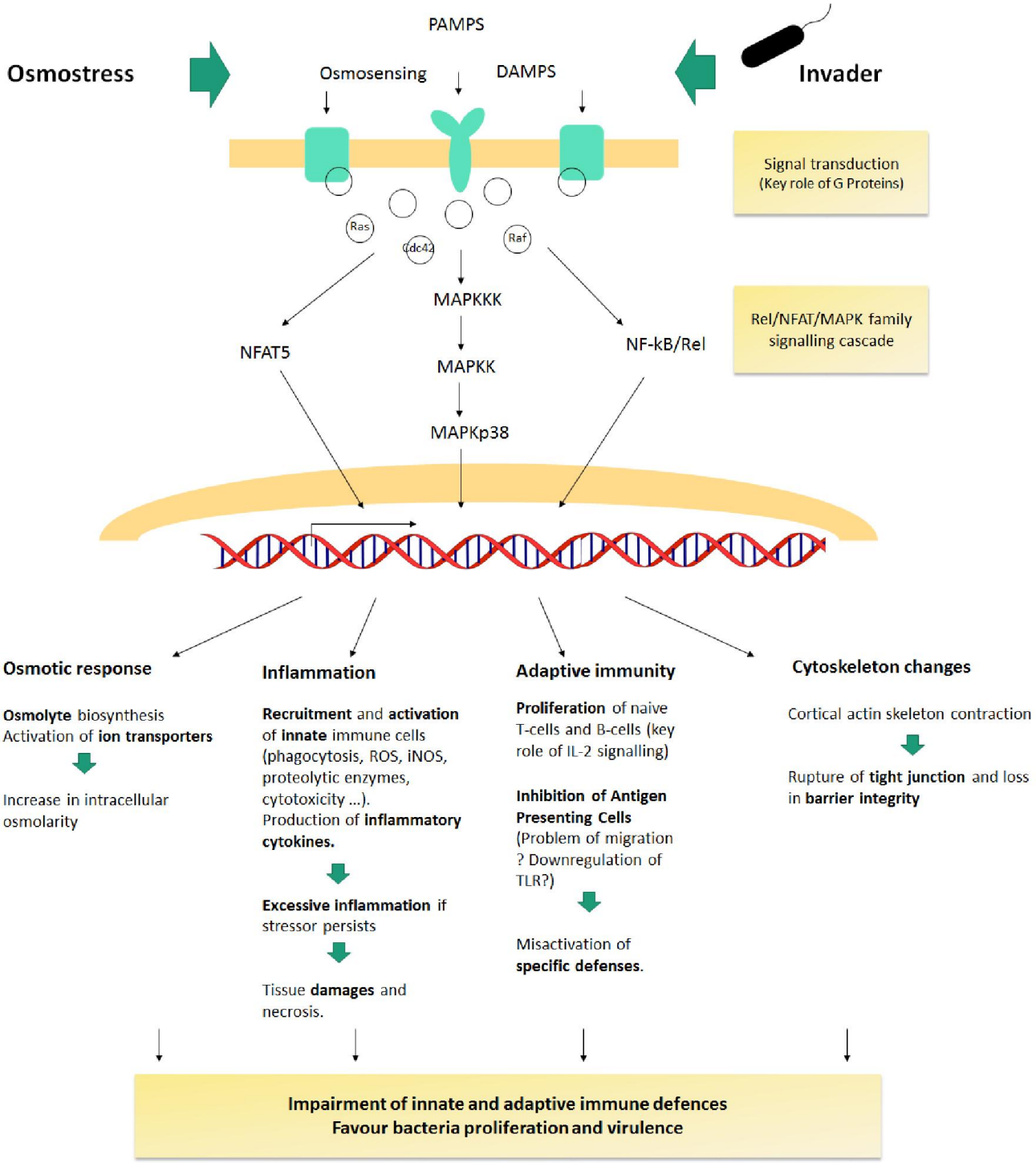


Figure 1: Hypothetic effects of chronic hyperosmotic stressor on the cellular and immune defences of striped catfish.

4. Which future for striped catfish farming?

In the Mekong Delta, climate changes threaten the livelihoods of striped catfish farmers operating in the lower reaches of the two main branches of the river (Nguyen et al, 2015). Several studies reported that downstream farms were suffering from lower yield, higher mortality rate, reduced growth rate and predicted that the net benefit in 2020 will be halved if no innovative ways to substantially reduce cost relative to climate changes are found (Phan et al., 2009; De Silva & Phuong, 2011; Kam et al., 2012). Salinities up to 10 ppt did not impair fertilization, hatching, larval development, growth rate and did not induce significant elevation of stress indicators (i.e. cortisol, glucose) in striped catfish (Do et al., 2012; Phuc et al., 2014). However, our results suggest that innate and adaptive immune parameters were enhanced/suppressed at salinities as low as 5 ppt although susceptibility to edwardsiellosis was not significantly affected below 20 ppt. Whereas inflammatory proteins, leukocytes abundance and activity were enhanced at elevated salinity, Toll-like receptors transcripts tend to decrease in kidney. In addition, our field studies revealed that salinity in pond was correlated with higher plasma osmolalilities in farmed catfish and that lysozyme activity and abundance of immune cells were higher in tidal farms during the dry season, when saline intrusions occurred. Therefore, we may hypothesize that seawater intrusions are already changing the immune status of striped catfish in Mekong Delta farms. Although freshwater pathogens might be also negatively affected by elevated salinities, prolonged higher plasma osmolarities may induce serious deleterious consequences such as tissue damage by chronic inflammation or slower specific immune response and eventually lead to decreasing immune health and lower resistance during disease outbreaks.

Furthermore, as mentioned above, a previous exposure to a stressor may affect the stress tolerance to other and therefore the efficiency of the immune system. In our study, high congestions have been observed on the opercular membrane, a key organ in aerial respiration and resistance to hypoxia (Lefevre et al., 2011a). Hence, it is likely that elevated salinity in ponds may affect hypoxia tolerance of striped catfish by decrease the capacity for aerial respiration. This is of particularly importance in hypoxic environment, such as typical Vietnamese catfish farms where oxygen level are inferior to 5 kPa (Lefevre et al., 2011b).

In the future, dams' construction in China, Laos and Thailand and increasing sea level rise will accentuate the problem of salinity in striped catfish farms. The current adaptation initiatives such as dike elevation or increase in water change are short term solutions that will not resolve in the long term the problems induced by seawater intrusions. Species switching to euryhaline tropical species would be a long term solution but will include significant changes in infrastructures and farming practises. Moreover, striped catfish farming success resides in its extraordinary capacity to resist to low level to oxygen, even in very high density and low water quality so species switching would likely induce additional costs such as pond aeration to maintain fish yield. Furthermore, striped catfish is native to the Mekong river and therefore species switching will include a high risk of introduction of non native species in the stream. An alternative solution to investigate resides in the selection of salinity tolerant strain of striped catfish that might be speeded up by the genomic selection technology.

5. Limitations of the thesis.

The use of non-common and non-model species (striped catfish, *Pangasianodon hypophthalmus*) induced several pitfalls and a significant risk of misinterpretation. First, laboratory and scientific tools such as western blot antibodies, primers in quantitative PCR, proteomic databases, KEGG pathway maps were all designed for common species for which the genome/proteome has been sequenced. In consequences, we had to work by homology with

more common species such as zebrafish or channel catfish, which was time-consuming, induced a significant unspecific filter, a significant loss of information and a risk to hybridize wrong proteins/transcripts in blotting techniques and quantitative PCR. Then, transportation of striped catfish from Thailand to Belgium induced more than 60% of mortality and therefore we involuntarily applied a first selection in term of stress resistance.

The main objectives of this thesis was to investigate the consequences of hyperosmotic stressors on fish basal and induced immune defences. It is crucial to keep in mind that we do not have injected control fish during the bacterial challenges, because of technical constraints in the containment laboratory (biosafety level 2), which comprises only three independent circuits. Therefore, it is not possible to isolate the effect of the bacterial infection from confounding factors such as the stress induced by the injection (which involved anesthesia, a short period of air exposure and the injection itself) as well as the transfer of infected fish in a new environment (containment facilities).

We choose to deepen immune responses in the kidney because in fish, kidney is the hematopoietic organ and contains melanomacrophage centers for antigen presentation. Moreover, kidney was the most affected organ during edwardsiellosis in term of number of bacterial nodules. However, immune responses are tissue-specific and therefore, our results might not be extrapolated to the whole organism. It may be interesting to investigate the effects of hyperosmolarity on other key actors of the immune system such as skin, mucus, gills, spleen and thymus.

Eventually, fish microbiota should be affected by elevated salinity. Therefore, change in immune parameters might be indirectly induced by change in absolute and/or relative abundance of microorganisms in fish organs and epithelia. Mucosal microbiota is particularly important in catfish species due to the absence of protective scales. The reasoning also applies to the virulent bacteria strain of *Edwardsiella ictaluri* used in this study. Increase in internal body osmolality may affect the propagation/virulence of the bacteria in catfish body, resulting on immune changes.

References

Buxadé, M., Lunazzi, G. , Minguillon, J. , Iborra, S. , Berga-Bolanos, R., Del Val, M. , Aramburu, J. , Lopez-Rodriguez, C. Gene expression induced by Toll-like receptors in macrophages requires the transcription factor NFAT5. *J. Exp. Med*. 2012, *209(2)*, 379-393.

De Silva, S., Phuong, N.T. Striped catfish farming in the Mekong Delta, Vietnam, a tumultuous path to a global success. *Rev. Aquacult*., 2011, *3,*45-73.

Do, T.T.H., Tran, N.T. The effect of salinity on the embryonic development and osmoregulatory of the striped catfish (*Pangasianodon hypophthalmus*) larvae and fingerling stages. *Journal of Science (CTU*), 2012, 21, 29–37.

DuBeau, S. F., Pan, F., Tremblay, G. C., & Bradley, T. M. Thermal shock of salmon in vivo induces the heat shock protein hsp 70 and confers protection against osmotic shock. A*quaculture*, 1998, *168*(1), 311-323.

Eckert, S.M., Yada, T., Shepherd, B.S., Stetson, M., Hirano, T., Grau, E.G. Hormonal control of osmoregulation in the channel catfish *Ictalurus punctatus. Gen. Comp. Endocr.,* 2001, *122,* 270–286.

Enyedi, B., Kala, S., Nikolich-Zugich, T., Niethammer, P. Tissue damage detection by osmotic surveillance. *Nat. Cell. Biol.,* 2013, *15*, 1123-1132.

Fan, H., Cook, J. A. Molecular mechanisms of endotoxin tolerance. *J. Endotoxin Res.,* 2004, *10***,** 71–84.

Hodgson, P. D., Aich, P., Manuja, A., Hokamp, K., Roche, F. M., Brinkman, F. S. L., ... , Griebel, P. J. Effect of stress on viral–bacterial synergy in bovine respiratory disease: novel

mechanisms to regulate inflammation. *Comparative and functional genomics*, 2005, *6*(4), 244-250.

Kam, S., Badjecjk, M., Tee, L., Tran, N. Autonomous adaptation to climate change bu shrimp and catfish farmers in Vietnam's Mekong River Delta. Worldfish, 2012.

Lefevre, S., Huong, D.T.T., Ha, N.T.K., Wang, T., Phuong, N.T., Bayley, M. A telemetry study of swimming depth and oxygen level in a Pangasius pond in the Mekong Delta. *Aquaculture*, 2011a, *315,* 410-413.

Lefevre, S., Jensen, F., Huong, D.T.T., Wang, T., Phuong, N.T., Bayley, M. Effects of nitrite exposure on functional haemoglobin levels, bimodal respiration, and swimming performance of the facultative air breather fish *Pangasianodon hypophthalmus*. *Aquat. Toxicol*., 2011b, *104*, 86-93.

Li, C. H., Wang, J. H., Redmond, H. P. Bacterial lipoprotein-induced self-tolerance and cross-tolerance to LPS are associated with reduced IRAK-1 expression and MyD88-IRAK complex formation. *Journal of leukocyte biology*, 2006, *79*(4), 867-875.

Neuhofer, W. Role of NFAT5 in inflammatory disorders associated with osmotic stress. Curr. Genomics, 2010, *11,* 584-590.

Norton, V., Davis, K. Effect of abrupt change in the salinity of the environmental on plasma electrolytes, urine, volume, volume and electrolytic excretion in channel catfish, *Ictalurus punctatus*. *Comp Biochem Phys. A*, 1977*, 56*, 425-431.

Novoa, B., Bowman, T. V., Zon, L., Figueras, A. LPS response and tolerance in the zebrafish (*Danio rerio*). *Fish & shellfish immunology*, 2009, *26*(2), 326-331.

Phan, L., Buy, T., Nguyen, T., Gooley, G., Ingram, B., Nguyen, H., Silva, S. Current status of farming practices of striped catfish, *Pangasianodon hypophthalmus*, in the Mekong Delta, Vietnam. *Aquaculture,* 2009, 296, 227-236.

Phuc, T., Huong, D., Mather, P., Hurwood, D.A. Experimental assessment of the effects of sublethal salinities on growth performance and stress in cultured tra catfish (*Pangasianodon hypophthalmus*). *Fish Physiol Biochem*, 2014, *40(6*), 1839-1848.

Pickering, A. D., & Pottinger, T. G. (1989). Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. *Fish physiology and biochemistry*, *7*(1-6), 253-258.

Pulsford, A. L., Lemaire-Gony, S., Tomlinson, M., Collingwood, N., Glynn, P. J. Effects of acute stress on the immune system of the dab, Limanda limanda. *Comp Biochem Physiol C*, 1994, *109*(2), 129-139.

Srivinas, S.P. Cell Signaling in Regulation of the Barrier Integrity of the Corneal Endothelium. *Exp. Eye Res*., 2012, *91,* 8-15.

Wang, J. H., Doyle, M., Manning, B. J., Blankson, S., Wu, Q. D., Power, C., Cahill, R., Redmond, H. P. Cutting edge: bacterial lipoprotein induces endotoxin-independent tolerance to septic shock. *J. Immunol.,* 2003, *170,* 14–18.

Chapter 8

General conclusions and perspectives.

This thesis aimed to investigate the immune responsiveness of striped catfish *Pangasianodon hypophthalmus* (Sauvage) to hyperosmotic stressors in environmental and husbandry conditions, as well as the possible consequences in disease sensitivity. This thesis runs along several lines including aquaculture, climate changes and allows better understanding the regulatory mechanisms behind adaptation to salinity, which have also many implications in human health.

Pertaining to our objectives, we conclude that striped catfish may be affected by hyperosmotic stressors including physiological, osmoregulatory and immune aspects as well as sensitivity to bacterial disease. Hyperosmotic stressors (low and high) were associated with changes in several physiological aspects such as decrease in the feed intake, development of congestion throughout the body, loss in tissue integrity, generation of key metabolite precursors and energy, stimulation of nucleic and proteic metabolisms and changes in lipid metabolism. Regarding osmoregulatory changes, hyperosmolarity elevated the internal body pressure, stimulated salt excretory pumps in gills and induced adaptation of renal excretory mechanisms. Regarding the immune status, we observed that innate immune cells as well as associated immune factors such as respiratory burst, lysozyme activity and phagocytosis were elevated in salt-stressed fish in normal condition and upon bacterial infection. On the contrary, we observed that despite the upregulation of key proteins involved in T-cells and B-cells regulation, the expression of Toll-like receptors was lowered in the kidney, especially during bacterial infection. Eventually, cumulative mortality upon infection with *Edwardsiella ictaluri* was not significantly affected by low salinity (up to 10 ppt) while susceptibility fastly increased in catfish faced to higher salinity (up to 20 ppt). Therefore, we hypothesized that hyperosmotic stressors was responsible for inflammatory response, whose intensity will vary depending on stressor intensity. Then, we hypothesized that specific immunity may be negatively affected by salinity and therefore may lead to higher sensitivity to pathogen.

However, further studies are needed to consolidate our hypothesis, especially regarding the specific immune response. For example, measurements of the abundance of cytokines, which orchestrate the inflammatory response, might be interesting. As the fish faced intracellular and extracellular increases in osmolality, the consequences of elevated salinity on the microbiota should also be investigated. Regarding proteomics, the complexity of whole proteomes exceeds the analytical capacity to isolate low-abundance proteins. Then, the depletion of high abundance proteins by, for example, subcellular fractionation or immunoaffinity column, may be useful to deplete very high-abundance proteins (e.g. cytoskeleton proteins) and enrich low-abundance proteins of interest (e.g. cytokines, chemokines, immune cells markers, major histocompatibility complex). In addition, we hypothesized that hyperosmotic stressors may interfere with the activation of adaptive immune cells and therefore the development of the specific immune response. However, these predictions are only based on expression ratio of some Toll-Like Receptors. Hence, it is essential to complement this first approach with other parameters such as the expression of major histocompatibility complex or the migration of sentinel cells in melanomacrophage centers by flow cytometry, immunohistochemistry or immunofluorescence techniques. These studies might be complemented with *in vitro* approaches. Indeed, we begun *in vitro* experiments

in order to investigate the effects of hyperosmotic extracellular media (270 mosm, 320 mosm, 360 mosm) with or without addition of retinoic acid on the morphology (nucleus, mitochondrial dispersion, actin cytoskeleton) and the immune activity (production of ROS, NOS, lysozyme, phagocytosis) of cultured peripherical mononuclear cells, combining confocal microscopy, biochemical techniques and flow cytometry. However, this experiment was not fully achieved because of technical problems and lack of time. It may be interesting to pursue this experiment, study supplementary parameters from the specific immunity and look for potential anti-inflammatory molecules in order to restore immune homeostasis in fish exposed to hyperosmotic stressors. These studies should also be extended in mammals regarding the wide use of hyperosmotic fluids in human health. Eventually, regarding our field studies, regular samplings associated with daily physicochemical data are needed in random striped catfish farms in order to extrapolate the future of striped catfish yield. Indeed, our *in vivo* experiment may depend on the particular rearing system and conditions used throughout the studies and therefore it is always difficult to extrapolate laboratory results to real environmental conditions.